

# PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : <b>C12Q 1/68, C12P 19/34</b>		(11) International Publication Number: <b>WO 92/15711</b>	
(21) International Application Number: <b>PCT/US92/01675</b>		(43) International Publication Date: <b>17 September 1992 (17.09.92)</b>	
(22) International Filing Date: <b>4 March 1992 (04.03.92)</b>		(74) Agent: HAMRE, Curtis, E.; Merchant, Gould, Smith, Edell, Weller & Schmidt, 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402 (US).	
(30) Priority data: 665,960 US 833,668 US 6 March 1991 (06.03.91) 18 February 1992 (18.02.92)		(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).	
(71) Applicant: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morril Hall, 100 Church Street S.E., Minneapolis, MN 55455 (US).		Published With international search report.	
(72) Inventors: SANTAMARIA, Pedro; 1716 Rollins Avenue SE, Minneapolis, MN 55414 (US). BOYCE-JACINO, Michael, Thomas; 1957 Berkeley Avenue, St. Paul, MN 55105 (US). BARBOSA, Jose, Joaquim; 1940 Westwood Circle, Roseville, MN 55113 (US). RICH, Stephen, Saul; 2266 Goodrich Avenue, St. Paul, MN 55105 (US). FARRAS, Anthony, James; 975 Tonkawa Road, Long Lake, MN 55356 (US).			

(54) Title: DNA SEQUENCE-BASED HLA TYPING METHOD

## (57) Abstract

The present invention provides a process for determining genotypes in highly polymorphic systems by polymerase chain reaction amplification of cDNA or genomic DNA and direct sequencing of the products. More specifically, Class II and Class I HLA genotypes can be unambiguously determined in any subject in 16-24 hours by direct sequencing of DRB, DQB, DQA, DPB, DPA, HLA-A, HLA-B and HLA-C transcripts enzymatically amplified using a limited number of non-allele-specific oligonucleotides. Total cellular RNA from peripheral blood mononuclear cells is reverse transcribed using antisense primers, specific for different loci (DRB1-3, or DQA, DPA, or DPB) or group of loci (DRB1-3, or HLA-A and HLA-B and HLA-C). The synthesized cDNA molecules are then enzymatically amplified using different combinations of oligonucleotides for each locus and directly sequenced with Taq polymerase using an internal oligonucleotide. The sequenced genes are then analyzed.

## PERIPHERAL BLOOD

### PRIMERS

### RNA

### cDNA

### PCR

### SPIN-DIALYSIS

### SEQUENCING

### ELECTROPHORESIS

### X-RAY FILM EXPOSURE

LOC1  
PRIMER #1  
REACTION

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

11 23 24 25 15 10 10 10 15 16

LOC2  
PRIMER #2  
REACTION

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

DRB DRB DRB DRB DQA DPA DPA

12 30 30 30 5 25 12 16 20

(A) (B) (C) (D) (E) (F) (G) (H) (I)

## FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BE	Belgium	GB	Great Britain	MR	Mauritania
BF	Burkina Faso	GD	Gabon	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BR	Brazil	HN	Honduras	NO	Norway
CA	Canada	IE	Ireland	PL	Poland
CH	Switzerland	IT	Italy	RO	Romania
CL	Chile	JP	Japan	RU	Russian Federation
CM	Cameroon	KE	Kenya	SE	Sweden
CN	China	KR	Republic of Korea	SG	Singapore
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LJ	Luxembourg	TG	Togo
ES	Spain	MC	Monaco	US	United States of America
		MG	Madagascar		

## DNA SEQUENCE-BASED HLA TYPING METHOD

## Technical Field

5 The present invention relates to a process for determining genotypes of highly polymorphic systems, such as the major histocompatibility complex of humans, including Class I and Class II HLA genes. Specifically, the method of the present invention involves amplifying the alleles carried by any given individual at a gene locus or loci of interest by polymerase chain reaction with conserved and non-conserved oligonucleotide primers. The polymerase chain reaction products are directly sequenced followed by evaluation of the resulting nucleic acid ladders to determine the genotype of sample nucleic acid.

## Background of the Invention

20 The major histocompatibility complex (MHC) includes the human leukocyte antigens (HLA) gene complex which is located on the short arm of human chromosome six. This region encodes cell-surface proteins which regulate the cell-cell interactions of the immune response. The various HLA Class I loci encode the HLA antigens, 44,000 dalton polypeptides which associate with B-2 microglobulin at the cell surface. The Class I molecules are involved in the recognition of target cells by cytotoxic T lymphocytes. HLA Class II loci encode cell surface heterodimers, composed of proteins of 29,000 and 34,000 daltons, respectively. These Class II molecules are also involved in the recognition of target cells by helper T lymphocytes.

30 The HLA-A, HLA-B, and HLA-C loci of the HLA Class I region as well as the HLA-DRB, HLA-DQB, HLA-DQA, HLA-DRB and HLA-DPA loci of the HLA Class II region exhibit an extremely high degree of polymorphism. The WHO nomenclature committee for factors of the HLA system

[Marsh and Bodmer, *Immunogenetics*, 31:131 (1990)] designated 25 alleles of HLA-A (HLA-A-0101, A-0201, etc.), 32 alleles of HLA-B, and 11 alleles of HLA-C, 43 HLA-DRB alleles, 13 HLA-DQB alleles, 8 HLA-DQA alleles, 5 4 HLA-DPA alleles and 19 HLA-DPB alleles. Since this high degree of polymorphism is thought to relate to the function of the HLA molecules, much effort has gone into determining its molecular basis and the functional implications of its polymorphisms (i.e., in transplantation). With the cloning of certain HLA genes this effort has extended to the DNA level.

10 The Class II genes of the HLA-D region on the short arm of human chromosome six constitute one of the most polymorphic genetic systems known [Bach, *Immunol. Today*, 5:89 (1985)]. The HLA Class II molecules (DR, DQ and DP) are heterodimeric glycoproteins composed of two non-covalently associated chains (alpha and beta) which serve as restricting elements in nominal antigen presentation in the context of self [Zinkernagel and Doherty, *Nature*, 248:701 (1974)] or as foreign antigens in alloresponses [Bach and Van Rood, *N. Engl. J. Med.*, 295:806 (1976)].

15 Allelic polymorphism of the HLA-D region encoded specificities can be determined by serological methods for phenotyping, mixed lymphocyte cultures using homozygous typing cells, primed lymphocyte testing, determination of restriction fragment length polymorphisms and, more recently, oligotyping [Bach, *supra* (1985); Bidwell, *Immunol. Today*, 9:18 (1988); Tiercy et al., *Proc. Natl. Acad. Sci. USA*, 85:198 (1988)]. Present efforts focus largely on the development of molecular approaches to typing, such as RFLP and oligotyping [Bidwell, *supra* (1988); Tiercy et al., *supra* (1988); Erlich and Bugawan, in PCR Techniques, H. A. Erlich, ed., Stockton Press, New York (1989)].

25 30 35

The cloning and sequencing of several HLA-DR, DQ, and -DP alleles has revealed that their amino acid polymorphisms are located in hypervariable regions of their N-terminal domains, encoded by the second exon of

5 DRB1, DRB3/4/5, DQA1 and DQB1, DPA1 and DPB1 genes [Marsh and Bodmer, supra (1990); Todd et al., Nature, 329:599 (1987)]. This information has allowed the

design of allele-specific oligonucleotides which can be used in the characterization of the known HLA Class II

10 polymorphisms by means of their hybridization to DNA on a solid support (oligomer typing) or for sequencing

[Tiercy et al., supra (1988); Erlich and Bugawan, supra, (1989); Todd et al., supra (1987); Saiki et al.,

15 Science, 230:1350 (1985); Mullis and Faloona, Methods Enzymol., 155:335 (1987); Saiki et al., Nature, 324:163

(1986); Scharf et al., Science, 233:1076 (1986); Gyllenstein and Erlich, Proc. Natl. Acad. Sci. USA,

85:7652 (1988)]. Oligonucleotide typing, although rapid, requires the use of a rather large number of

20 oligonucleotides for each locus and cannot detect

previously unidentified sequence polymorphisms, likely to exist in non-Caucasian populations; further, the approach may not be easily applicable to and may not be practical for the analysis of Class I polymorphisms.

25 Direct sequencing of single-stranded DNA generated by

PCR using allele-specific oligonucleotides has been successfully used to examine polymorphism at DQA1 locus [Gyllenstein and Erlich, supra (1988)]. Application of

30 this approach to DRB genes is, however, problematic due to the strong sequence homology among DRB1, DRB3, DRB4 and DRB5 genes and the presence of up to four different versions of each of these genes in most individuals (isotypic complexity). The very complex ladders

35 generated by direct sequencing make this present process impractical for accurate and rapid determination of HLA types. Thus, direct sequencing of HLA-PCR products has been limited to previous knowledge of the HLA types

carried by a given individual and as such is not suitable for routine HLA typing [Bach, supra (1985); Bidwell, supra (1988); Tiercy et al., supra (1988); Erlich and Bugawan, supra (1989)].

5 Currently, HLA typing is routinely done in connection with many medical procedures, e.g., organ transplantation. Rejection of organ grafts is believed to be diminished if the HLA alleles of donor and recipient are identical. The numerous alleles of HLA genes in the population also make HLA typing useful for

10 paternity testing. However, the currently available techniques are incapable of differentiating among all of the polymorphisms associated with the alleles at Class I

and Class II HLA loci. Other drawbacks to current HLA

15 typing are the availability of standard sera necessary to conduct serological tests, the speed of obtaining test results (i.e., MLC takes 5-7 days), and that only the already known HLA types, but not new polymorphisms, are detected by these techniques. In the case of tissue

20 typing in organ transplants and in relatively high volume genetic evaluations, such as paternity testing, the length of time associated with current HLA typing techniques causes unnecessary delay and the results may not be highly accurate.

25 Accordingly, there is a need for a method to determine genomic information in highly polymorphic systems, such as the HLA gene complex, that addresses the limitations imposed by previous methods. That is, in the case of the HLA gene complex, a system that is capable of determining the nucleotide sequences of the genes carried by any given individual without the need to have previous knowledge of his or her HLA types as defined by other methods. Furthermore, the invention avoids the use of oligonucleotides specific for each known allele. The technique we present is rapid, requires the use of only a small number of

oligonucleotide primers, and can readily detect new

sequence variants unidentifiable with more conventional approaches. This system is exemplified by its applicability to the analysis of Class II as well as Class I and Class III genes and is automatable.

5

#### Summary of the Invention

The present invention relates to a method for determining the nucleic acid sequence of one or more polymorphic genes of a subject by amplifying and direct sequencing genomic or complementary DNA molecules for each allele at each gene locus to be sequenced using conserved and non-conserved (non-allele-specific) oligonucleotide primers. In a broad sense, the method of the present invention involves sequence-based typing (SBT) which provides for unequivocal determination of genetic polymorphism at any genetic locus of interest by direct, simultaneous, sequence analysis of both genomic DNA or expressed (RNA) copies of such a locus. SBT can be employed to determine genetic polymorphism at one or more genetic loci of interest, regardless of the complexity of the polymorphism at these loci, including, for example: (1) simple homozygosity or heterozygosity of a unique locus, as exemplified by DQA or the like; (2) isotypic complexity due to multiple, closely related and closely linked copies of a locus, as exemplified by DRB or the like; and (3) intra-allelic complexity at a locus compounded by interlocus complexity, such as Class I genes or the like. Most known human genetic polymorphisms are of the first, and simplest, type.

Use of the SBT method provides overlapping sequence data comprised of only the copies of the locus of interest as is exemplified by each of the types of HLA loci. The SBT strategy is designed to ensure selection of a given locus with equal representation of each copy of that locus by equal amplification and direct sequencing of mixtures of both alleles of that locus and direct interpretation of the overlapping

sequencing patterns generated by this approach. Thus, providing a method for determining genetic polymorphism at one or more genetic loci of interest which can be employed, for example, in HLA typing, detection, evaluation, and/or characterization of genetic diseases such as, for example, sickle cell anemia, cystic fibrosis, Thalassemia, and the like, and detection, evaluation, and/or characterization of polymorphism in genetic loci associated with various cancers such as p53, Ras, myc, associated with carcinomas, leukemias, sarcomas or the like.

Use of the method according to the present invention is exemplified by a system providing for rapid and accurate determination of a major histocompatibility complex class genotype of a subject in a sample (e.g., Class I or Class II). Most particularly, the method is directed to determining at least one HLA Class II gene locus including DRB1, DRB3, DRB4, DRB5, DOB1, DQA1, DPA1 and DPB1 genes. In the case of Class I genotypes, the method is envisioned as being useful to determine A, B, and C loci genes.

To determine a gene locus nucleic acid sequence polymorphism with the method of the present invention, nucleic acid (RNA or DNA) from a sample is isolated. In the case of RNA, cDNA molecules for each allele of at least one gene locus to be sequenced are synthesized by employing a locus-specific oligonucleotide primer that anneals to a conserved region of each allele of each gene locus. According to the present invention, the sample nucleic acid sequence is determined by:

amplifying the cDNA molecules or genomic DNA by polymerase chain reaction to generate sufficient product for each allele of each gene locus to be sequenced, with all of the alleles for each gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer pair, and at least one of the alleles of each gene locus and chromosome to be

sequenced being amplified with at least one non-conserved oligonucleotide primer and at least one conserved primer; preparing the products of each PCR for sequencing (clean); sequencing directly the products of each polymerase chain reaction product to detect each allele at each gene locus of each chromosome, with an enzyme appropriate for DNA sequencing, such as Taq polymerase and a conserved primer specific for each locus that is sequenced; and analyzing each sequenced product for each locus and primer combination(s) to determine the genotype of the subject.

In a preferred embodiment of the present invention the sequence of each polymerase chain reaction product for each allele of each gene locus is determined by analyzing each nucleic acid single and/or overlapping ladder generated for each directly sequenced polymerase chain reaction product. The analysis is conducted by comparing the nucleotide sequence of each allele of each gene locus sequence to known sequences for each locus, followed by comparing the sequence of each gene locus amplified with the non-conserved/conserved oligonucleotide primer pair to the nucleotide sequence of each allele of the gene locus amplified with a conserved oligonucleotide primer pair. Comparison of nucleic acid ladders for sequenced alleles can be conducted visually or using computer software.

In a preferred embodiment, the process of the invention is automated for use in rapid genotype determinations, including diagnosis of genetic diseases. Automation of the process includes isolating the sample nucleic acid with an RNA/DNA extractor; amplifying the synthesized cDNA molecule or the isolated DNA molecule by polymerase chain reaction using a thermocycler to generate the polymerase chain reaction products; sequencing the polymerase chain reaction products in an automated sequencing apparatus; and analyzing each sequenced polymerase chain reaction product with the

computer having a database with allelic sequence information and the capacity to conduct the appropriate subtraction algorithm for comparing the polymerase chain reaction product sequence for each allele amplified with a conserved oligonucleotide primer pair to the nucleic acid sequence of each allele sequenced with a non-conserved/conserved oligonucleotide primer pair.

The invention further relates to specific groups of oligonucleotide primers useful in the steps of cDNA synthesis, cDNA/genomic DNA amplification by polymerase chain reaction and direct sequencing of the polymerase chain reaction products to determine the nucleotide sequence of each of the alleles at each locus of each chromosome that is amplified. Useful single strand DNA oligonucleotide primers are described in Table 1 herein.

#### Brief Description of the Drawings

Figure 1A shows a schematic of the cDNA/PCR/Sequencing experiments for DRB (DRB1, DRB3, DRB4 and DRB5), DQA1, DQB1, DPAl and DPB1 genes.

Figure 1B shows a schematic of the primer binding sites on DRB, DQA1, DQB1, DPAl and DPB1 transcripts. Stippled boxes represent primers used in the cDNA synthesis reactions; black boxes represent conserved (or Type 1) primers, used for PCR; checked boxed represent non-conserved (or Type 2) primers, also used for PCR; and blank boxes represent sequencing primers.

Figure 1C shows a schematic of the primer binding sites on DQB1, DRB, DPB1 and DPAl genes in their germline configuration. Only those primers exclusively used for genomic DNA samples are shown in the Figure.

Figure 2A shows a flow-chart of the procedure for peripheral blood samples. Each reaction is performed in a different test tube. The reactions are

named with capital letter in parenthesis; these letters correspond to those shown in Table II (combinations of primers/reaction). Only the "routine" combinations of primers are shown in this Figure.

Figure 2B is a flow-chart of the procedure for forensic samples, where DNA is usually the only available genetic material to work with. DNA in these situations is usually isolated from hair, sperm, blood stains, etc. The combinations of primers per reaction shown in the Figure correspond to the "routine" combinations only.

Figure 3 shows direct sequencing of Class II HLA dsDNA generated using conserved oligonucleotides.

Lanes are read from left to right as G-A-T-C. 1, DQB1 ladder for a DQB1\*0201/DQB1\*0302 heterozygote; 2, DQA1 ladder for a DQA1\*0103 homozygous cell line; 3, DRB ladders for a DRB1\*0301, DRB3\*0101/DRB1\*0401, DRB4\*0101 heterozygote. Positions where there is more than one band are indicated on the side of the ladder and the templates they correspond to are indicated at the top of the Figure. To read unambiguously the last 50-60 base pairs of the ladder it is necessary to electrophorese the sequencing gel for an additional hour. Note that the ladders corresponding to the genes at DRB3 or DRB4 loci are fainter in comparison to those corresponding to the genes at DRB1 locus, possibly due to their lower levels of expression. These differences in intensity are generally reproducible and help read the complex overlapping patterns. The positions of the first exon base pair and codon (in parenthesis) that can be read in this Figure are indicated at the bottom of each ladder.

Figure 4 shows direct sequencing of Class II HLA DRB1 dsDNA generated using non-conserved oligonucleotides. Lanes are read from left to right as G-A-T-C. Lane 1, DRB1\*0101/DRB1\*1501, DRB5\*0101 heterozygote cDNA amplified with primer DRB17 (selects DRB5\*0101 cDNA) (left) and DRB16 (selects DRB1\*0101

cDNA) (right); lane 2, DRB1\*1405, DRB4\*0101/DRB1\*0301, DRB3\*0101 heterozygote cDNAs amplified with the 5' primers DRB17 (selects DRB1\*0301 and DRB3\*0101 cDNAs) (left) and DRB16 (selects DRB1\*0405 and DRB4\*0101) (right). Positions where there is more than one band or where the two ladders generated with each primer differ are indicated on the side of the ladders.

#### Detailed Description of the Invention

As used herein, the term "gene" refers to a segment of DNA, composed of a transcribed region and a regulatory sequence that makes possible a transcription. The term "gene locus" refers to the specific place on the chromosome where a gene is located. The term "allele" refers to the multiple forms of a gene that can exist at a single gene locus at a single chromosome and are distinguishable from the other possible alleles by their differing effects on phenotype (detectable outward manifestations of a specific genotype). "Haplotype" refers to the specific allele composition of the genes at multiple loci on the same chromosome. As used herein the term "genotype" refers to the specific allelic composition of a gene at multiple linked loci at each chromosome (2 haplotypes).

The term "oligonucleotide" as used herein refers to a molecule having two or more deoxyribonucleotides or ribonucleotides, preferably more than three deoxyribonucleotides. The exact number of nucleotides in the molecule will depend on the function of the specific oligonucleotide molecule. As used herein the term "primer" refers to a single stranded DNA oligonucleotide sequence, preferably produced synthetically which is capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to a nucleic acid strand to be copied or a point of initiation for sequencing a DNA molecule. In the case of primers intended for use in

synthesizing cDNA or amplifying cDNA or genomic DNA molecules by polymerase chain reaction products, the length and sequence of the primer must be sufficient to prime the synthesis of extension products in the presence of a polymerization enzyme. Preferably, the length of the primer is from about 5-50 nucleotides, more preferably from about 5-20 nucleotides. Specific length and sequence of the primer will depend on complexity of required DNA or RNA target templates, as well as conditions of primer employment such as temperature, ionic strength, and MgCl<sub>2</sub> concentration. When nested primers are used for sequencing, the number of base pairs separating the amplification and sequencing primers on the DNA template are also important considerations.

As used herein, "conserved oligonucleotide primer" (Type 1) refers to an oligonucleotide molecule that corresponds to a region of high DNA sequence conservation (i.e. less than 1-2 nucleotide variations). While the conserved primer need not correspond exactly to the nucleotide template to which it anneals, the conserved primer will have minimal, preferably less than one mismatch with the target nucleotide template. Functionally, conserved primers are capable of equally priming the target nucleotide (cDNA, PCR product, etc.) at high stringency conditions. In contrast to this, as used herein, "non-conserved oligonucleotide primer" (Type 2) refers to an oligonucleotide molecule that has an intended number of mismatches with the possible target nucleotide sequences. The intended number of mismatches can vary with a preferred number of mismatches being about 1-12. Non-conserved primers are characterized by their selective binding to a limited number of alleles at a given locus or at a group of highly homologous loci. The non-conserved primer will bind to the more complementary allele or group of alleles (two or less than two) (i.e., fewer number of

mismatches between primer and target template sequence). The specific combinations of conserved and non-conserved primers and the number of reactions per locus or loci used herein are specifically designed to obtain highly accurate results with minimal expenditure of time and cost.

The present invention is directed to a process for determining the sequences of the alleles of polymorphic gene systems carried by any given individual, such as, for example, the human HLA system, genes related to different human genetic disorders, such as sickle cell anemia, cystic fibrosis, or the like, as well as gene systems associated with various cancers, such as p53, myc, or the like. The present invention is exemplified by its utility for determining polymorphism at HLA loci, particularly Class II and Class I genes, the most polymorphic human genetic loci known today, using enzymatic amplification and direct sequencing of the gene cDNA molecules using a limited number of primers and avoiding the use of allele specific oligonucleotides as much as possible. The present method is particularly well suited to determining allelic sequences of Class II HLA genes, thereby providing complete HLA Class II genotype information for a subject. Using the method of the present invention complete Class II HLA typing (DR, DQ and DP) can be performed in about 16 to 24 hours or less.

Generally, the method of the present invention involves: extraction of sample nucleic acid; in the case of RNA, generation of cDNA; cDNA or genomic DNA amplification; direct sequencing of amplification products; and analysis of the direct sequence information. Generation of cDNA, amplifying the cDNA and direct sequencing the cDNA amplification products is accomplished using oligonucleotide primers with specific characteristics, such as those described herein.

### A. Oligonucleotide Primers

The oligonucleotide primers of the present invention can be synthesized using any known suitable method, such as phosphotriester and phosphodiester methods. Narang et al., Methods Enzymol., 53:90 (1979); Brown et al., Methods Enzymol., 58:109 (1979). Oligonucleotides can be prepared using a modified solid support such as a Biosearch 8750 DNA synthesizer. Useful primers can also be isolated from a biological source using appropriate restriction endonucleases which cut double stranded DNA at or near a nucleotide sequence of interest for use as a primer.

### B. Extraction of Sample Nucleic Acid

In the process of the present invention any source of nucleic acid can be used as the sample nucleic acid, as long as the sample contains the nucleic acid sequence of interest. For example, the sample chosen for the present method can be RNA, DNA or a DNA/RNA hybrid. Typical samples include peripheral blood mononuclear cells, (PBMC's), lymphoblastoid cell lines (LCL's), hair cells or the like. For determining human HLA Class II and Class I gene polymorphisms LCL's or PBMC's are preferred. The nucleic acid to be isolated (e.g. RNA or DNA) will depend on the source of genetic material (blood stain, hair, or peripheral blood cells). However, in the case of HLA Class II genes including DRB1-5, DQB1, DQA1, DPAl, DPE1 the preferred isolated nucleic acid is total cellular RNA when the typing is to be done for transplantation purposes or paternity testing. For forensic uses, genomic DNA may be the preferred genetic material in which case different primer considerations would be used. Cytoplasmic and poly(A) + RNA can also be used. It is envisioned that isolation of sample nucleic acid for the present process can be automated using a DNA/RNA extractor (such as

Model 341 DNA extractor available from Applied Biosystems, Inc.; Foster City, CA).

### C. Generation of cDNA

Complementary DNA (cDNA) of the sample nucleic acid is generated using specific oligonucleotide primers and cloned reverse transcriptase following general conditions suggested by the enzyme manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). Specific differences in type and amount of primers used, dNTP concentrations and elongation times will be readily apparent to those of skill in the art based on the Examples that follow.

### D. Polymerase Chain Reaction

Amplification of cDNA or genomic DNA for each gene locus of interest is accomplished using the polymerase chain reaction (PCR) as generally described in U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis. The PCR consists of many repetitions of a cycle which consists of: (a) a denaturation step, which melts both strands of a DNA molecule; (b) an annealing step, which is aimed at allowing the primers to anneal specifically to the melted strands of the DNA molecule; and (c) an extension step, which incorporates to the primers deoxyribonucleotides complementary to those of the strand of DNA to which the primers are annealed. The PCR process, as indicated in the Examples, can be conducted using a Thermocycler (Perkin-Elmer, Cetus, Emeryville, CA).

The present invention introduces the use of non-conserved oligonucleotides in the PCR procedure specifically designed to solve the problems associated with, for example, detecting, evaluating, and/or characterizing polymorphism at a polymorphic gene locus or loci of an individual. In the case of HLA typing, the use of non-conserved oligonucleotides addresses the

problems one would face in performing HLA typing by sequencing DNA amplified exclusively by using conserved oligonucleotides or allele specific oligonucleotides (see below).

5 It is understood that the PCR process is designed for the amplification of specific genes with the use of oligonucleotides specific for the particular gene to be amplified. However, even using completely matched primers, in most cases the PCR is not absolutely specific. In the case of HLA typing, for HLA-DRB genes and Class I genes, the use of conserved primers in PCR will generate complex mixtures of templates, which upon direct sequencing will be seen as overlapping sequencing ladders, cumbersome to interpret. Therefore, genes for which the exact nucleotide sequence information is unknown can not be achieved with an adequate level of certainty. Use of non-conserved oligonucleotides which can selectively anneal under high stringency conditions to two or fewer alleles of a gene locus or group of homologous loci can provide sequence information for the different genes at highly homologous loci in complex heterozygote combinations. Thus, the present invention provides a method useful for determining the genotype for polymorphic gene loci. This is of particular importance to HLA typing, and is applicable to Class I HLA typing as well as Class II typing.

The difference between non-conserved primers and allele-specific oligonucleotides resides in that the latter can only be used when the presence of a particular allele is known, and also requires the use of a specific primer for each of the alleles of the polymorphic system. Thus, combining use of a non-conserved primer and conserved primers to amplify the separate alleles of highly homologous polymorphic gene loci can provide simpler DNA polymerase chain reaction product combinations sufficient to allow unambiguous interpretation of direct sequencing ladders of each

allele for genotype determinations with moderate expenditure of time and economical cost.

The conditions used for the PCR reactions are preferably the same except for the temperature used in the annealing step, which is different depending on the type of primer used, conserved (Type 1) or non-conserved (Type 2). Reactions that use the former primer type are preferably performed at 37°C in the annealing step of the cycle, whereas this step is preferably performed at about 55°C to 60°C in reactions that use the later type of primers. The concentrations of primers, and buffers used will be apparent from and include the process parameters described in the Examples that follow.

#### 15 E. Direct Sequencing Of PCR Products

Direct sequencing of double-stranded DNA generated by the PCR is accomplished using an enzyme appropriate to DNA sequencing, such as Taq polymerase, or the like, and specific combinations of reagents at appropriate concentrations. The sequencing procedure can be conducted in an automatic sequencing apparatus such as the 373A Model DNA Sequencer from Applied Biosystems Inc. (Foster City, CA). The reagents, including sequencing primers and nucleic acid termination mixtures will be understood by those of skill in the art based on the direct sequencing procedure specified in the following Examples.

#### F. Analysis Of Direct Sequenced PCR Products

The nucleic acid ladders resulting from direct sequencing the cDNA or genomic DNA for each gene locus of interest can be assessed visually from autoradiograms or by employing a computer programmed with nucleotides sequence information for all alleles of all haplotypes and procedures for comparing sequenced alleles and known alleles of gene loci of interest. In a preferred embodiment of the present invention, the evaluation of

gene locus alleles involves a two step process: (a) comparison of the gene sequences of each polymerase chain reaction product (i.e., conserved and non-conserved primer products) with a library of known genotype information such as the information obtained on homologous cell lines very well characterized by methods other than sequencing [Marsh and Bodmer, Immunogenetics, 31:131 (1990)] as well as sequences of individual alleles; followed by (b) comparison of direct sequence information for the polymerase chain reaction product of an allele of a gene locus amplified with a conserved oligonucleotide primer pair and polymerase chain reaction product of alleles of a gene locus or loci amplified with a conserved/non-conserved primer pair.

15 This comparison employs a substitution algorithm or visual cancellation of duplicative sequence ladder information to generate the specific sequence information for each allele of a gene locus.

It is envisioned that the process of the present invention can be used to amplify and sequence known and unknown highly polymorphic systems (e.g., genetic disease-related genes, cancer-related genes, and HLA typing, including Class I, Class II, and Class III HLA typing, and the like). The present process is believed to be useful for paternity testing and forensic medicine, with more accuracy than restriction fragment length polymorphism (RFLP), DNA fingerprinting or dot blot-detection systems. While in the latter only a hybridization pattern is observed, direct sequencing of amplified products shows the exact nucleotide sequence of the amplified genes, and hence is more accurate and reliable.

The method is particularly well suited for Class II HLA typing, reducing its costs, increasing its speed and especially improving its accuracy. As evidenced by the following Examples, sequence polymorphism analysis of DRB1, DRB3, DRB4, DRB5, DQB1,

DQA1, DPAl and DPB1 genes can be rapidly performed in any subject of unknown HLA type by means of enzymatic amplification and direct sequencing of Class II genes using a limited number of conserved and non-conserved oligonucleotides. The approach described herein is entirely automatable using currently available technology and, as opposed to previously described methods using oligonucleotide probes and dot blots, has the advantage of detecting the presence of new allelic sequences or sequence microheterogeneity at the population level. The methodology of present invention is envisioned to be useful for detailed analyses of the effects of sequence allelism at different Class II HLA loci on graft survival after allogeneic transplantation. The method of the present invention allows rapid and precise sequence analysis of Class II HLA polymorphism in studies of human disease and may be of interest in the search for new Class II sequence variants in large populations of subjects.

The present invention is further described by illustration in the following Examples which are not intended to limit the invention.

#### EXAMPLE I

##### 1. Preparation of Oligodeoxyribonucleotide Primers and Sequence Primer Combinations Useful for cDNA/PCR/Sequencing Reactions of Class II HLA Genes

All of the oligodeoxyribonucleotide primers described herewithin were synthesized as described below:

Automated Synthesis of oligodeoxyribonucleotide primers: The b-cyanoethylphosphoramidites, obtained from Milligen-Bioscience (Novato, CA), were sequentially condensed to a nucleoside derivatized controlled pore glass support using a Bioscience 8750 DNA synthesizer. Condensation cycles included detritylation with dichloroacetic acid in dichloromethane, followed by condensation with benzotriazole and capping with acetic

anhydride and 1-methylimidazole in tetrahydrofuran and pyridine, with each cycle time being approximately 9 minutes. Yields at each step were >99% as determined by measuring dimethoxytrityl alcohol release. The methodology for oligodeoxyribonucleotide synthesis is described in Caruthers, et al., Methods Enzymol., 154:287 (1987).

## Deprotection and purification of

oligodeoxyribonucleotide primers: Deprotection and  
purification of oligodeoxyribonucleotide primers was  
performed using the procedure described by Schulhof et  
al., Nucl. Acids Res., 15:397 (1987). Briefly, the  
oligodeoxyribonucleotide was removed from the solid  
support by exposure to concentrated ammonium hydroxide  
at room temperature for about one hour. The solution  
containing the partially deprotected

oligodeoxyribonucleotide was brought to 65°C for 16 hours. Ammonia was removed and the residue was subjected to chromatography on a C18 reverse-phase column (RP 304, BiORad, Richmond, VA) using a linear gradient of 14 to 20% acetonitrile in 0.1 molar ammonium/triethylamine, pH 7.0. The dimethoxytrityls group was removed from the HPLC-purified

oligodeoxyribonucleotide by treatment with 70% acetic acid. The detritylated oligodeoxyribonucleotide was recovered by precipitation in ether, vacuum centrifuged until dry, resuspended in water and quantitated by measuring its absorbance at 260nm.

Using the above procedure, the following oligonucleotide primers corresponding to specified regions of HLA Class II DQA, DQB, DRB, DPB and DPA loci were synthesized (see Table I below) and extensively tested:

TABLE I  
Oligonucleotides Used For The cDNA/PCR/Sequencing Reactions

No.	Listing (Seq.)	Type 1	Anneal	Locus (1)	Template	Step
1	DQB7	5'-GGTGGTGAAGGCGCTGTGTCC-3'	105-111	DQB1	RNA	RT/PCR
2	DQA9	5'-GGTGAAGGTTACTATCTTGTAAAC-3'	105-111	DQA1	RNA	RT/PCR
3	DQB13	5'-AGACACTCTCTCCGAGGATTTTC-3'	1-7	DQB1	RNA	PCR/SEQ
4	DQB12	5'-CTGGCTTTTGGCTGGGGAACAC-3'	-4-3	DQB1	RNA/DNA	PCR/SEQ
5	DQB22	5'-CTGGCTTTTGGCTGGGGAACAC-3'	-4-3	DQB1	RNA/DNA	PCR/SEQ
6	DQB11	5'-TGTTCTCCAGGATGGTGTGTGC-3'	-33/-26	DQB1	RNA	PCR
7	DQA10	5'-CTGGCTCTCCGATGATGAAGCC-3'	-10/-4	DQA1	RNA	PCR
8	DQB932	5'-TGGCCCTCTGGCAGGGTTCGGGCG-3'	88-94	DQA1	DNA	PCR
9	DQB931	5'-TTTAAGGCGATGTTGCTTAC-3'	11-17	DQB1	DNA	PCR
10	DQB30	5'-ATGGGATGATGGTACTGTGG-3'	97-104	DQB1	RNA	SEQ
11	DQB30	5'-AGGATTAACATGCTCCCTTGG-3'	97-103	DQB1	RNA	SEQ
12	DQB5	5'-CTAGTTGTGTGTGGTGGACAC-3'	78-83	DQB1	RNA/DNA	SEQ
13	DQB12	5'-GCCGCTGCACTGTGAGAGTTC-3'	87-94	DQB1	RNA	SEQ
14	DQA29	5'-CAGGGTTCCGGTGAACAGCGGTAG-3'	82-89	DQA1	RNA	SEQ
15	DQA30	5'-ATCGGTTCCCTCTTGGCCAG-3'	19-24	DQA1	RNA	SEQ
16	DQB1400	5'-GCCCTTTCAGCAGCGCAAGTGG-3'	38-45	DQB1	RNA/DNA	SEQ
17	DQB1401	5'-GAGGTGTACTGTGTATCTCTGAC-3'	98-104	DQB1	RNA/DNA	PCR
18	DQB1402	5'-GATCAAGGCTGGGACACAC-3'	142-148	DQB1	RNA/DNA	PCR
19	DQB1403	5'-CCGGAACGCGTGAAGCTTCAAT-3'	127-133	DQB1	RNA/DNA	SEQ
20	DQB1406	5'-GCCAAGAGTGGGCGCTTCGACAC-3'	bp18-38	DQB1	DNA	PCR
21	DQB25	5'-AACCCCGTAGTGTGTGTGCA-3'	79-85	DQB1	DNA	SEQ
22	DQB24	5'-GGGGAACACCGACACGTTTC-3'	-1-7	DQB1	DNA	PCR
23	DQB10	5'-CAGCAGTGGCTGTCTGACGGGCG-3'	-19/-13	DQB1	RNA	PCR
24	DQB11	5'-GTTGTGTGTGCTGTGCAGAGGCGCC-3'	105-111	DQB1	RNA	RT/PCR

21

Sequence Listing (Seq.)	No. Type 1	Sequence Listing (Seq.)	No. Type 2
DPB12	25	5'-CTTGAGGAGGAGAAACATTCAC-3'	42
DPB13	26	5'-TACTGATGTGTGCTGCTCAGAT-3'	43
DPB14	27	5'-AGAGGAGAGAGAGAGATTCAG-3'	44
DPB15	28	5'-GCCCTGCGGACAGGCGCCCGG-3'	45
DPB16	29	5'-CGGCCCAAGAGCGCTCAGTCA-3'	46
DPB17	30	5'-CGCTCATGTGTGCGCCCTCC-3'	47
DPB18	31	5'-GTCAATGTGTGAGATGAGGCT-3'	48
DPB19	32	5'-CATATCATAGAGCTGTGATCTG-3'	49
DPB20	33	5'-CTTGAGGAGAGAGAGAGATTCAG-3'	50
DPB21	34	5'-CTGCTGAGATGTGCTCCAGAGCT-3'	51
DPB22	35	5'-CTGCTGAGATGTGCTCCAGAGCT-3'	52
DPB23	36	5'-AGCTGAGAGGCTGCGCAGAGAG-3'	53
DPB24	37	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	54
DPB25	38	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	55
DPB26	39	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	56
DPB27	40	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	57
DPB28	41	5'-AACTTGAAATACCTTGATCCAG-3'	58

Sequence Listing (Seq.)	No. Type 1	Sequence Listing (Seq.)	No. Type 2
DPB12	25	5'-CTTGAGGAGGAGAAACATTCAC-3'	42
DPB13	26	5'-TACTGATGTGTGCTGCTCAGAT-3'	43
DPB14	27	5'-AGAGGAGAGAGAGATTCAG-3'	44
DPB15	28	5'-GCCCTGCGGACAGGCGCCCGG-3'	45
DPB16	29	5'-CGGCCCAAGAGCGCTCAGTCA-3'	46
DPB17	30	5'-CGCTCATGTGTGCGCCCTCC-3'	47
DPB18	31	5'-GTCAATGTGTGAGATGAGGCT-3'	48
DPB19	32	5'-CATATCATAGAGCTGTGATCTG-3'	49
DPB20	33	5'-CTTGAGGAGAGAGAGATTCAG-3'	50
DPB21	34	5'-CTGCTGAGATGTGCTCCAGAGCT-3'	51
DPB22	35	5'-CTGCTGAGATGTGCTCCAGAGCT-3'	52
DPB23	36	5'-AGCTGAGAGGCTGCGCAGAGAG-3'	53
DPB24	37	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	54
DPB25	38	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	55
DPB26	39	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	56
DPB27	40	5'-GGCTGAGAGGCTGCGCAGAGAG-3'	57
DPB28	41	5'-AACTTGAAATACCTTGATCCAG-3'	58

All the above Type 1 primers are annealed at 37°C and the Type 2 primers are annealed at 55°C. When the later anneal at 37°C in the PCR, they do not distinguish among allelic transcripts differing by few base pairs. This list of primers includes primers which are only used in certain situations, performed at the other linked loci. The alternative combinations of primers used in each step are described in Table II below. (\*) These primers anneal to a polymorphic region of DBI cDNAs (codons -8 to -2) encoding the 3' end of the signal peptide which has specific nucleotide sequences for different DBI alleles (DQB6-DQB1\*0601 and DQB1\*0604-, DQB14-DQB1\*0501-, DQB15-DQB1\*0301-). RT = Reverse transcription; Seq = sequencing.

22

## 2. Combinations of Primers for cDNA/PCR/Sequence Reactions

There are specific combinations of oligonucleotide primers for each reaction and for each locus, including cDNA synthesis, PCR amplification and direct sequencing, which are designed to provide all the necessary sequence information for obtaining highly accurate, fast and inexpensive typing results. These combinations are listed in Table II below as "routine" combinations. In addition, Table II includes a list of "alternative" combinations of oligonucleotides for each locus which may be used to confirm results obtained with the "routine" combinations for a particular locus not expected according to, for instance, known haplotypic maps. These "unexpected" results are usually indicative of the existence of new alleles and/or haplotypes, which can be confirmed with the use of the alternative combinations of oligonucleotides. In any case, each of these combinations of oligonucleotides is characterized by its ability to generate an end-product (sequencing ladder) which is suitable of being accurately read by the naked eye or processed by computer operated under appropriate software.

For typing purposes in the clinical setting, such as in transplantation, the method uses RNA isolated from peripheral blood mononuclear cells as starting material; for forensic purposes, however, DNA is often the only available template. Although for each template (RNA or DNA) different combinations of oligonucleotides are used (see Table II), the general strategy for typing, including the interpretation of the results is essentially the same. The specific combinations of primers for "routine" RNA and DNA analysis, respectively, are described below in more detail. The general overview of the HLA typing strategy is shown in Figures 1 and 2 and discussed further in Examples 2 and 3.

TABLE II  
Combinations of Primers for cDNA/PCR/Seq Reactions

1. RNA				
Type	CDNA	PCR	A.T.	Seq*
<b>Routine</b>				
A. 1	DRB20	DRB11	37°C	
DRB30/DRB12/DRB22**				DRB30/DRB12
B. 2	DRB20	DRB23	55°C	DRB30/DRB12
C. 2	DRB20	DRB24	55°C	DRB30/DRB12
D. 2	DRB20	DRB25	55°C	DRB30/DRB12
E. 1	DOB7	DOB13	37°C	DOB30/DOB5
F. 1	DOA9	DOA10	37°C	DOA30/DOA29***
G. 1	DPB11	DPB10	37°C	DPB12/DPB13
H. 1	DPA14	DPA15	37°C	DPA16/DPA17
I. 1	DPA19	DPA18	37°C	DPA20/DPA21****
<b>Alternative</b>				
J. 2	DRB20	DRB16	55°C	DRB30/DRB12
K. 2	DRB20	DRB17	55°C	DRB30/DRB12
L. 1	DRB20	DRB22	37°C	DRB30/DRB12
M. 1	DOB7	DOB6	37°C	DOB13
N. 2	DOB7	DOB6	55°C	DOB30/DOB5
O. 2	DOB7	DOB14	55°C	DOB30/DOB5
P. 2	DOB7	DOB15	55°C	DOB30/DOB5
Q. 1	DPB12	DPB10	37°C	DPB13
R. 1	DPA16	DPA15	37°C	DPA17
<b>2. DNA</b>				
Type	PCR1	PCR2	A.T.	Seq
<b>Routine</b>				
S. 1	DRB1406	DRB22	37°C	
DRB12/DRB1400*****				DRB12/DRB1400
T. 2	DRB1406	DRB24	55°C	DRB12/DRB1400
U. 2	DRB1406	DRB25	55°C	DRB12/DRB1400
V. 2	DRB1406	DRB23	55°C	DOB5
W. 1	DOB332	DOB931	37°C	DOB5
X. 1	DPB14	DPB15	37°C	DPB16/DPB17
Y. 1	DPA10	DPA11	37°C	DPA12
<b>Alternative</b>				
Z. 1	DRB12	DRB24	37°C	DRB825
AA. 2	DRB1401	DRB1402	55°C	DRB1403#
AB. 2	DRB1406	DRB16	55°C	DRB825/DRB12
AC. 2	DRB1406	DRB17	55°C	DRB825/DRB12
AD. 1	DPB14	DPB16	37°C	DPB17

(\*) For sequencing DRB and DOB two alternative sequencing primers are indicated, both sequencing the positive strand of DNA.

25

(\*\*) Primer DRB22 is used to sequence the negative strand whenever new allelic sequences are identified.

(\*\*\*) Each DQA1 sequencing primer anneals to a different strand. Reaction L uses an alternative amplification primer (DRB 22 instead of DRB11) in hypothetical situations where homozygosity may not be expected according to the rest of the haplotype. Reaction M is used for sequencing the negative strand of DQA1 in situations where new allelic sequences are identified.

(\*\*\*\*) Sequencing of the third exon is necessary to distinguish among certain DPAl alleles.

(\*\*\*\*\*) Primer DRB1400 may be used in sequencing amplified DRB genes from genomic DNA to read the sequences immediately following the 3' amplification primer. Reaction Q, R, Z and AD are alternative combinations for confirming homozygosity at the corresponding loci which may not be expected according to the rest of the Class II haplotype.

(#) This primer combination is used to distinguish between DRB1\*0701 and DRB1\*0702, which differ by a single base pair in their third exons.

26

## EXAMPLE II

Protocol: HLA Class II "Typing" by Direct Sequencing of DRB, DQB, DQA, DPA and DPB Genes

## 1. Cell Lines and Subjects

5 Lymphoblastoid cell lines (LCLs) representing each of the known Class II haplotypes defined at the 10th International Histocompatibility Workshop [Dupont, Hum. Immunol., 26, 3 (1989)] were provided by Dr. Miriam Segall (University of Minnesota). Forty unrelated

10 subjects who had been previously serologically typed for Class I and Class II antigens were also studied. The serological types of each of the subjects under study were not known to the investigator performing the sequence analysis at the time the analysis was

15 performed. These subjects included both healthy and affected (insulin-dependent diabetes and autoimmune thyroid disease) individuals. The sequenced haplotypes,

many in heterozygote combinations, included: DR7 (n=3), DRW17 (n=26), DR4 (n=16), DRW11 (n=8), DRW8 (n=4), DR1 (n=6), DRW15 (n=6), DRW16 (n=2), DRW13 (n=2), DRW14 (n=2), DR212 (n=3), DR5x6 (n=3). The cell lines and heterozygote combinations tested are shown in Table III.

Since the complexity at DPA and DPB loci is similar to that of DQ genes, the primer combinations for DPA and

25 DPB typing were optimized in a smaller group of homozygote and heterozygote subjects.

## 2. HLA-DRB, DQB and DQA Transcript Amplification Using Conserved and Non-Conserved Oligonucleotides

30 Total cellular RNA was prepared from (1 µg)

from 5-10x10<sup>6</sup> peripheral blood mononuclear cells (PBMC) or lymphoblastoid cell lines (LCLs) by cesium chloride centrifugation [Chirgwin et al. Biochemistry, 18, 5249

(1979)]. Alternatively, total RNA from peripheral blood (2-10 ml) was partially purified using a much faster

35 protocol [Gouh, Anal. Biochem., 173, 93 (1988)]. One microgram of total cellular RNA was reverse transcribed with Moloney leukemia virus reverse transcriptase

(MIVRT) (200 u, Bethesda Research Laboratories) in 50 mM Tris HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3mM MgCl<sub>2</sub>, in the presence of the ribonuclease inhibitor RNAs in (10 units, Promega), 75  $\mu$ M each dNTP and 10 pmols of a specific non-sense primer (Table II) in a 20 ml final volume for 30-45 min at 37°C. Eight  $\mu$ l of 10X PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3, 7.5-15 mM MgCl<sub>2</sub>, 0.1% gelatin) were added after the incubation period. A 5'-primer (20 pmols) (Type I or Type 2

10 primers, respectively, see Table II) plus 10 pmols of the non-sense primer and two units of Taq polymerase

were also added and the final volume was adjusted to 100  $\mu$ l with distilled water. The reaction mixture was subjected to 35 cycles of 30 sec at 94°C, 30 sec at 37°C or 55°C and 30 sec at 72°C using a Perkin-Elmer Cetus Thermocycler [see Saiki et al., *supra* (1985); Mullis and Faloona, *supra* (1987); Saiki et al., *supra* (1986);

Scharf et al., *supra* (1986)]. The primers used here, their corresponding sequences and the regions to which they anneal are shown in Table II. The reactions for each locus are usually performed in separate microfuge tubes. However, when using conserved primers, the cDNA and PCR reactions for all loci (DRB, DQA, DQB, DPA and DPB) can be successfully performed simultaneously in the same tube.

### 3. Direct Sequencing of Amplified Products with Taq Polymerase

The reaction mixture (100  $\mu$ l) was freed of unincorporated dNTPs and excess of oligonucleotides by spin-dialysis using Centricon-100 (Amicon) or Ultrafree-100 (millipore) microconcentrators. One half of the retentate (20  $\mu$ l) was dried down and resuspended in 15  $\mu$ l of 1X Taq sequencing buffer (50 mM Tris-HCl, pH 9, 10 mM MgCl<sub>2</sub>). Internal oligonucleotides were used for priming the sequencing of DQB, DRB, DQA, DPB and DPA genes, respectively (Table II). Primers for sequencing each strand are listed in Table II. Only one strand is

routinely sequenced for typing; sequencing of the other strand is performed in cases where a new allelic sequence is suspected. Eighty to 100 ng of primer were end-labelled with 10 pmol of gamma-P32 labelled ATP

5 (5000 Ci/mmol, 10  $\mu$ CI/ $\mu$ l) and 5 units of T4 polynucleotide kinase (Promega Biotec) in a 10  $\mu$ l final volume. Ten ng of primer (1  $\mu$ l) were added to the sequencing mixture without extraction of unincorporated labelled ATP, boiled for 5 min., and then left at room temperature for 15 min. Eight units of recombinant Taq polymerase (USB) were added to the mixture. Four  $\mu$ l of the annealed primer/template mixture were later added to 4  $\mu$ l of each of the stop nucleotide mixes: a) Term mix

ddG: 15 microM each dGTP, dATP, dCTP, dTTP; 45 microM ddGTP; b) Term mix dda: 15 microM each dGTP, dATP, dCTP, dTTP, dTTP; 600 microM ddATP; c) Term mix ddt: 15 microM each dGTP, dATP, dCTP, dTTP; 1200 microM ddCTP; d) Term mix ddc: 15 microM each dGTP, dATP, dCTP, dTTP; 450 microM ddCTP. The reactions were allowed to proceed for

20 two consecutive periods of 10 min. at 72-74°C. After the second cycle, each reaction was chased with 2  $\mu$ l of a 7.5  $\mu$ M mixture of ATP, GTP, TTP, CTP, and allowed to

proceed for 5 min. After spinning down, the reaction was stopped by adding 4 ml of 95% (vol/vol) formamide/20 mM EDTA, heated to 80°C for 5 min. and loaded on a .4 mm thick 6% polyacrylamide/7M urea gel. Electrophoresis was performed at 2500 V for 2 hr, the gel fixed in 5% (vol/vol) glacial acetic acid/5% (v/v) methanol for 15 min, dried, and exposed to Kodak X-Omat film for 4 to 12 hours.

### RESULTS

#### 1. Sequence-Based Typing of DR and DQ Polymorphic Genes in Homozygous Typing Cells

35 Homozygous lymphoblastoid cell lines (LCLs)

from the panel of the 10th International

Histocompatibility Workshop (Table III) were used as an initial test of the methodology. In total, these cell

29

lines were representative of most of the known DR and DQ alleles at the time the analysis was conducted.

Total cellular RNA isolated from homozygous

5 LCLs was reverse-transcribed and the resultant cDNAs amplified using conserved oligonucleotides specific for DRB1/DRB3/DRB4/DRB5 or DQB1 or DQAI genes as described in the preceding protocol. The conserved or Type 1

oligonucleotide primers anneal to regions of conserved DNA sequences; these regions are identical among the

10 known alleles at each locus and flank the second exon of Class II genes. These conserved primers, as opposed to non-conserved or Type 2 primers, are designed to amplify all known alleles at DRB, DQAI and DQB1 loci and, thus, all possible combinations of these alleles in any given

15 heterozygote. The Type 1 oligonucleotides did not cross-amplify templates at loci other than those

specified by the oligonucleotides (i.e., the DQAI

primers did not amplify DRB or DQB1 transcripts and vice versa); as expected, the DRB primers also amplified any 20 DRB3, DRB4 or DRB5 transcripts present in addition to DRB1. Sequencing of these amplified templates was

performed using a Type 1 primer annealing to a conserved region of the cDNAs internal to the sequence recognized by the amplification primers. Figure 1A shows the

25 general strategy for the method (SBT) and Figure 1B shows the relative position of each of the

oligonucleotides used for the cDNA, PCR and sequencing reactions on the mature DRB, DQA and DQB mRNA molecules.

The sequences of these primers, the loci they are

30 specific for, the specific positions (codons) to which they anneal and the reaction(s) they are used in are

indicated in Table II where the specific combinations of primers that can be used for the cDNA/PCR/sequencing reactions for each locus are identified. As noted in

35 the legend to Table II, some of the primer combinations shown represent alternatives which may be useful in confirming results for a particular locus which do not

30

fit with the expected sequences usually found with the rest of the haplotype. Each cDNA/PCR reaction is

usually performed in a separate tube. However, when

using Type 1 primers, the cDNA/PCR reactions for all the

5 loci (DRB, DQA, DQB, DPA and DPB) can be performed simultaneously in the same tube. The products of each

locus are sequenced in separate tubes. Following the

conditions described in the above protocol, the sequence ladders between the sequencing primer and the 5'

10 amplification primer could be clearly read starting from 2 to 14 bases from the sequencing primer binding site.

No anomalous amplification products or sequencing

ladders were detectable upon direct sequence analysis of amplified DRB, DQB1 and DQAI cDNAs from the 43

15 homozygous cell lines tested (Table IIIa). The specific alleles at each Class II HLA locus composing the haplotypes carried by each of these cell lines are shown in Table IIb. The number of ladders generated for each cell line was always that expected according to the

20 specificity of the amplification primers (one DQB1 and one DQAI ladder for all cell lines, one DRB ladder for DR1 and DRw8 cell lines and two DRB ladders for

haplotypes of the DRw52 and DRw53 supertypic groups).

Thus, analysis of the homozygous typing cell lines 25 showed that the Type 1 primers used for cDNA synthesis, PCR and

sequencing reactions allowed for accurate amplification and sequencing of all the tested alleles at each of these loci.

TABLE IIIA

## Cell Lines and Heterozygote Combinations Tested

Cell Line	Class II HLA Type	Subject	Class II HLA Types**
SA	DR1-Dw1	S1	DR1-Dw1/DRw17
M2070782	DR1-Dw20	S2	DR1-Dw1/DRw4-Dw4
KAS011	DRw16-Dw21	S3	DR1-Dw1/DRw8.1
*CALOGERO	DRw16-Dw-	S4	DRw15-Dw2/DRw17
*WJR076	DRw16-Dw21	S5	DRw15-Dw2/DR4-
Dw4			
*DEM	DRw16-Dw21/DR4	S6	DRw16-Dw21/DRw17
WT24	DRw16-Dw21	S7	DR5x68/DRw17
RML	DRw16-Dw22	S8	*DRw13-
Dw18/DRw17			
SCHU	DRw15-Dw2	S9	DRw13-Dw19/DRw17
WT8	DRw15-Dw2	S10	DR4-Dw4/DRw17
*AMAI	DRw15-Dw2	S11	DR4-Dw4/DRw12
E4181324	DRw15-Dw12	S12	DR4-Dw13/DR1-Dw1
MT14B	DR4-Dw14	S13	DR4-Dw13/DRw17
EJ32B	DRw17-SYD3	S14	DR4-Dw14/DRw15-
Dw2			
RSH	DRw18-DwRSH	S15	*DR4-Dw15/DRw17
DEU	DR4-Dw4	S16	DRw11-Dw5/DRw17
WT51	DR4-Dw4	S17	DRw12/DR1-Dw1
JBAF	DR4-Dw13	S18	DRw12/DRw8.1
YAR	DR4-Dw10	S19	*DRw14-Dw9/DRw17
KT17	DR4-Dw12	S20	DR7/DRw17
SPOO10	DRw11-DB2	S21	DR4-Dw4/DR7
JBUSH	DRw11-Dw5	S22	DRw8.1/DR7
TISI	DRw11-DwTISI	S23	DRw8.1/DR5x68
JVM	DRw11-DwJVM	S24	DRw8.2/DRw11-Dw5
BM16	DRw12-DB6	S25	DRw8.3/DR1-Dw1
*H0301	DRw13-Dw19	S26	DRw8.3/DRw15-Dw2
WDV	DRw13-Dw18	S27	DR9/DR1-Dw1
WT47	DRw13-Dw19		
TEM	DRw14-Dw9		
EK	DRw14-Dw9		
ANALA	DRw14-Dw16		
LBF	DR7-DB1		
BH	DR7-DB1		
CF96	DR7-Dw7		
BER	DR7-Dw7		
DBB	DR7-Dw11		
MOU	DR7-Dw17		
BTB	DRw8-Dw8.1		
OLGA	DRw8-Dw8.2		
LUY	DRw8-Dw8.3		
TAB089	DRw8-Dw8.3		
DKB	DR9-Dw23		

The allelic composition at DRB, DQA1 and DQB1 loci for the sequenced haplotypes corresponded to that expected according to published sequence information from well

characterized homozygous cell lines unless indicated (\*).

\* Haplotypes carrying new allelic sequences (DRB1, DRB3, DQA1 or DQB1 loci).

\* Only the tested heterozygote combinations are listed. The remainder of the 40 subjects tested were homozygotes or carried the haplotypes listed in this table.

§ This DRB specificity (DR5x6) has been given this arbitrary designation according to serological, RFLP and sequence information.

Table IIib  
Allelic Composition of Human Class II Haplotypes

Haplotype	YS#	DRB1	DRB3	DRB4	DRB5	DOA1	DOA1
DR1-Dw1	9001	*0101	-	-	-	*0501	*0101
DR1-Dw20	9002	*0102	-	-	-	*0501g	*0101
DR16-Dw21	9009,-84,-15	*1601	-	-	*0201	*0502	*0102
DR16-Dw21	9012,9007	*1601	-	-	*0202	*0502	*0102
DR16-Dw22	9016	*1602	-	-	*0201	*0501	*0501
DR15-Dw2	9013,9017	*1501	-	-	*0101	*0602	*0102
DR15-Dw2	9010	*1501	-	-	-1,3	*0602	*0102
DR15-Dw12	9011	*1502	-	-	*0102	*0601	*0103
DR17-Dw3	9088	*0301	*0101	-	-	*0201	*1501
DR17-DwSYD	9085	*0301	*0201	-	-	*0201	*0501
DR18-DwRSH	9021	*0302	*0101	-	-	*0402	*0401
DR4-Dw4	9029	*0401	-	*0101	-	*0301	*0301
DR4-Dw13	9030	*0403	-	*0101	-	*0302	*0301
DR4-Dw13	9024	*0403/6	-	*0101	-	*0301	*0301
DR4-Dw10	9026	*0402	-	*0101	-	*0302	*0301
DR4-Dw14	9028	*0404	-	*0101	-	*0302	*0301
DR4-Dw15	KT3 (a)	*0405	-	*0101	-	*0401	*0301
# -	CC	*0405	-	*0101	-	*0201	*0301
DR11-DB2	9036	*1104	*0201	-	-	*0502	*0102
DR11-Dw5	9035	*1101	*0201	-	-	*0301	*0501
DR11-Dw5	9035	*1101	*0201	-	-	*0301	*0501
DR11-DwJVM	9039	*1102	*0201	-	-	*0301	*0501
DR12-DB6	9038	*1201	*0201	-	-	*0301	*0501
# -	EX8	DR5X5	*0101	-	-	*0301	*0501
DR13-Dw19	9055	*1302	*0301	-	-	DQ8.5	*0102
DR13-Dw18 (b)	9062	*1301	*0101	-	-	*0503	*0103
DR13-Dw19	9063	*1301	*0101	-	-	*0502	*0102
DR13-Dw19	9057,9054	*1302	*0301	-	-	*0604	*0102
DR14-Dw9	9057,9054	*1401	*0201	-	-	*0503	DOA1.4
# -	MA	*1401	*0201	-	-	DQ85.4	ND
DR14-Dw15	9064	*1402	*0101	-	-	*0301	*0501
DR7-DB1	9096,9046	*0701	-	*0101	-	*0201	*0201
DR7-Dw7	9094,9093	*0701	-	*0101	-	*0201	*0201
DR7-Dw11	9052	*0701	-	*0101g	-	*0303	*0201
DR7-Dw17	9050	*0701	-	*0101	-	*0201	*0201
DR8-Dw8.1	9067	*0801	-	-	-	*0402	*0401
DR8-Dw8.2	9071	*0802	-	-	-	*0402	*0401
DR8-Dw8.3	9070	*0803	-	-	-	*0301	*0601
DR8-Dw8.3	9066	*0803	-	-	-	*0501	*0103
DR9-Dw23	9075	*0901	-	-	-	*0303	*0301
DR10 (c-e)		*1001	-	*0101	-	*0501	*0101

The allelic composition at DRB, DOA1 and DOB1 loci for the sequenced haplotypes corresponded to that expected according to published sequence information from well characterized homozygous cell lines unless indicated (\*).

\* Haplotypes carrying new allelic sequences (DRB1, DRB3, DOA1 or DOB1 loci).

\* Only the tested heterozygote combinations are listed. The remainder of the 40 subjects tested were homozygotes or carried the haplotypes listed in this table.

g This DRB specificity (DR5x6) has been given this arbitrary designation according to serological, RFLP and sequence information.

## 2. Amplification and Direct Sequencing of DQA1 and DQB1 cDNAs in Subjects of Unknown HLA Type

DNA sequences have been determined for most HLA Class II allelic specificities defined by conventional HLA typing techniques (March, S.G.E., Bodmer, J.G. HLA-DRB nucleotide sequences, 1990. Immunogenetics 31:141, 1990; Todd, J.A., Bell, J.I., McDevitt, H.O.: HLA-DQB gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329:599, 1987). Comparisons of these sequences indicates that any given DQA1 or DQB1 homozygous or heterozygous allelic combination is characterized by a specific sequencing ladder.

Total RNA from PBMCs from 40 different

subjects was tested to evaluate if the allelic composition of DQA1 and DQB1 homo- and heterozygotes could be determined correctly by direct amplification and sequencing using Type 1 primers. These subjects had been previously serologically typed but the typing information was not known to the investigator who

assigned the Class II allelic specificities from the sequencing results. These 40 subjects comprised 27

different heterozygote combinations (Table III). All individuals were assigned DQA1 and DQB1 allelic

sequences that were consistent with the serological phenotypes. In all the heterozygotes tested, both

allelic sequences could be read clearly from the composite sequence pattern. A unique pattern is found

for every particular heterozygote combination in the same way that certain RFLP banding patterns correspond

to certain heterozygote allelic combinations. For

instance, in a DQB2/DQB1.1 heterozygote one would find the sequence GGG(A/T)T(T/A)CCGGC(A/G) at codons 45 to

49 which can only be attributed to that particular

allele combination. In practice, interpretation of heterozygous sequence ladders is initiated by reading

certain polymorphic positions where allele-specific bases may be found, such as, for instance, the second

base of codon 46, where DQB1\*0201 is the only allele that has an A. The sequences of the two possible templates are then deduced and compared with the sequences of all known alleles at the different loci.

In Figure 3 we show the overlapping ladder corresponding to a DQB1\*0201/DQB1\*0302 heterozygote; interpretation of the pattern is indicated on the side of the ladder.

The absence of expected bands or the presence of unexpected bands for a particular allele or allelic combination is therefore suggestive of sequence heterogeneity, i.e., new alleles. The same can be said for DPA1 and DPB1 typing when appropriate primer combinations are used (Table III). For instance, substitution of the A at the second base of codon 46 would strongly suggest the presence of a sequence variant of DQB1\*0201. Once detected, the sequence of the variant can be confirmed after selective amplification of the variant or by subcloning the amplified products.

## 3. Amplification and Direct Sequencing of DRB cDNAs from Subjects of Unknown HLA Type

As described above, the use of Type 1 primers

allows the unambiguous sequencing of all heterozygous

combinations of DQA1 and DQB1 alleles. The same can be said for DPA1 and DPB1 typing when appropriate primer

combinations are used (Table II). Because of the

isotypic complexity of DRB genes (expression of more than one DRB locus by certain haplotypes), amplification

and sequencing of cDNAs from DRB heterozygotes with Type 1 primers can generate up to four overlapping ladders,

thus generating complex sequencing patterns.

DRB cDNAs from the same 40 individual tested

above for DQA1 and DQB1 genes were amplified and

sequenced using DRB-specific Type 1 primers. As

mentioned above, these 40 individuals comprised 27

different heterozygote combinations, including several examples from each of the groups of complex DRB allelic

combinations which would generate up to four sequencing ladders. The DRB sequence ladders generated with Type 1 primers were analyzed as described above for DQA1 and DQB1 loci: highly polymorphic positions were analyzed first for the presence of bands unique to specific alleles or groups of alleles (i.e., DR4) and the sequences deduced and compared with the sequences of all known alleles at all loci. As example, in Figure 3 we show the ladder generated by sequencing a complex DRB heterozygote (four overlapping ladders); the positions with two or more bands are indicated on the side of the Figure and assigned to each of the allelic types composing the complex sequencing pattern. For all but one sample, the information deduced from these sequencing experiments matched the independently determined serological phenotypes of the subject under study as well as the DQA1 and DQB1 allelic types assigned to these individuals by direct sequencing of these genes as described above. The inconsistent sample had been serologically typed as DRw13/DR4 but was typed by sequence analysis as DRw13/DRw8-Dw8.1. The presence of a DRB1\*0801 allele instead of a DRB1\*0401 allele was confirmed in a repeated experiment; we thus believe that the serological typing was in error. In all the 40 cases, all DQB1, DQA1 and DRB1 templates had been equally amplified and sequenced with a similar efficiency by the use of Type 1 primers. DRB3, DRB4 and DRB5 sequence ladders could be read in all but one case (a DRB3\*0101 [DRw52a] sequence was not initially observed in a DRw13/DRw17 heterozygote). Since DRB3\*0101 is in linkage disequilibrium with DRB1\*0301, the former allele was expected to be found in the overlapping ladder as well. In order to rule out the possibility of an error, the investigator assigning the HLA types from the sequencing ladders repeated the typing of this individual; the DRB3\*0101 could be read in the repeated experiment.

Although the results generated by the use of Type 1 primers were compatible with the serological phenotypes, the exclusive use of Type 1 primers will not allow in all cases to assign each of the specific ladders to each of the expressed loci in all possible heterozygotes. Given below are the most complex situations which cannot be addressed by the exclusive use of Type 1 primers: 1) distinction among the different DR4 allelic sequences in certain heterozygotes since they differ by only a few nucleotide base pairs and such differences could be masked by the presence of additional ladders; 2) to distinguish between DRB1\*1601 and DRB1\*1502 since their sequence differences will be masked by those of their linked DRB5 alleles; 3) to distinguish between DRB1\*1301 and DRB1\*1302 (which only differ at codon 86 since this difference can also be masked by other ladders; and finally 4) distinction between DRB1\*0301 and DRB1\*0302 in specific heterozygote combinations.

We have thus developed a more informative strategy to deal with DRB; this strategy, which consists of the additional use of non-conserved (Type 2) primers permits the clear elucidation of even the most complex combination of the four DRB sequences that might be present in an individual. These non-conserved primers, as opposed to allele-specific primers, are designed to be used in reactions performed simultaneously with the reactions using Type 1 primers and aim at selectively amplifying certain ladders from the complex sequencing patterns without requiring previous typing information.

Analysis of the sequence variability of the second exon of the DRB genes has allowed us to identify two regions which could be used to design non-conserved (Type 2) primers: 1) codons 5-13; and 2) codons 29-35. The sequence of the former region follows a group-specific sequence pattern, i.e., a sequence shared by groups of alleles at individual loci. The latter region

exhibits a scattered nucleotide polymorphism in DRB1 and DRB3, DRB4 and DRB5 genes. We designed five different non-conserved primers annealing to these two polymorphic regions: 1) DRB23 (specific for DR2-DRB1 ladders); 2) DRB24 (specific for DRw17-, DRw18-, DRw13-, DRw14-, DRw11-, DRw12-, and DRw8- DRB1 ladders); 3) DRB25 (specific for DR4- DRB1 ladders); 4) DRB16 and 5) DRB17, the latter two primers annealing to the second region of moderate polymorphism (from 1 to 5 nucleotides different among the known alleles for each locus) (Tables I and IV). Because of the different nature and distribution of mismatches between these primers and the different DRB templates, the type of templates amplified selectively by these primers will be different. Each of the first three primers will amplify up to two DRB1

CDNAs in any given heterozygote and will not amplify any DRB3, DRB4 or DRB5 cDNAs. On the contrary, the use of primers DRB16 and DRB17 will allow the random selective amplification of certain transcripts from DRB1, DRB3, DRB4 and/or DRB5 loci in most heterozygote combinations. We therefore tested these primers in order to determine which combination would give the best discriminatory results for DRB typing. Furthermore, since the sequences of these primers carry from 0-12 mismatches with the sequences of the known DRB alleles at the different DRB loci, their use allowed us to determine the number of mismatches between the primers and each of the possible cDNAs that are required to obtain such selective amplification of DRB transcripts. The specific combinations of primers used for the cDNA/PCR/sequencing reactions are shown in Table II above. The results of this analysis are shown below.

Table IIIa. Mismatches between Type 2 DRB-primers and DRB alleles at different loci.

Contribution of Nucleotide Base Pair Mismatches Between 5' Amplification Primers and DRB Alleles to the Selective Amplification of Allelic And/or Non-Allelic DRB Transcripts		DRB1	
		*1601-2	*1401-2
DRB16	0	2	4
DRB17	4	0	7
DRB23	5	0	0
DRB24	6	5	8
DRB25	6	4	8
		DRB5#	
DRB16	1	3	6
DRB17	4	1	5
DRB23	5	4	5
DRB24	6	6	5
DRB25	6	8	6
		DRB3/DRB4/DRB5	
		DRB3*0101	DRB3*0201
DRB16	3	4	5
DRB17	4	3	5
DRB23	2	4	5
DRB24	8	8	5
DRB25	8	8	5
		DRB3*0301	
DRB16	3	4	5
DRB17	4	3	5
DRB23	2	4	5
DRB24	8	8	5
DRB25	8	8	5
		DRB4	
DRB16	4	2	4
DRB17	8	3	2
DRB23	12	8	4
DRB24	12	12	4
DRB25	12	12	4

# DRB5 gene from cell line AMAT has an additional nucleotide substitution in the first base of codon 30, in comparison with DRB5 genes of other DR2 haplotypes.

# The DRB1 gene of this specificity (DR5x6) has been given this arbitrary designation according to serological, RFLP and sequence information.

TABLE V

Selective Amplification of DRB and DQB1 cDNAs  
in Combinations of Alleles Mismatched with  
Type 2 Oligonucleotides(†)

Haplotypes	Selected Alleles	DO Primer
DRB1*1301, DRB3*0101, DRB1*1601, DRB5*0201	DRB1*1601	DRB16
DRB1*1301, DRB3*0101, DRB1*0801	DRB1*0801	DRB16
DRB1*0301, DRB3*0101, DRB1*1601, DRB5*0201	DRB1*1601	DRB16
DR5*6, DRB3*0101, DRB1*0801	DRB1*0801	DRB16
DR5*6, DRB3*0101, DRB1*0801	DRB3*0101	DRB17
DR5*6, DRB3*0101, DRB1*0801	DR5*6	DRB16
DRB1*1101, DRB3*0201, DRB1*1501, DRB5*0101	DRB3*0201	DRB16
DRB1*1101, DRB3*0201, DRB1*1501, DRB5*0101	DRB5*0101	DRB17*
DRB1*1201, DRB3*0201, DRB1*1101, DRB3*0201	DRB1*1101	DRB16
DRB1*1201, DRB3*0201, DRB1*1101, DRB3*0201	DRB1*1201 + DRB3*0201	DRB17
DRB1*0405, DRB4*0101, DRB1*0301, DRB3*0101	DRB1*0405 + DRB4*0101	DRB16
DR5*6, DRB3*0101, DRB1*1101, DRB3*0201	DRB1*1101 + DR5*6	DRB16
DRB1*1501, DRB5*0101	DRB1*1501	DRB16
DRB1*1601, DRB5*0201, DRB1*0401, DRB4*0101	DRB1*0401 + DRB5*0201	DRB17
DRB1*1601, DRB5*0201, DRB1*0401, DRB4*0101	DRB1*1601 + DRB1*0401	DRB16**
DRB1*1601, DRB5*0201	DRB1*1601	DRB16
DRB1*1601, DRB5*0201	DRB5*0201	DRB17
DRB1*1602, DRB5*0202	DRB1*1602	DRB16
DRB1*0401, DRB4*0101	DRB4*0101	DRB16
DQB1*0604, DQB1*0502	DQB1*0604	DQB6
DQB1*0301, DQB1*0101	DQB1*0301	DQB6
DQB1*0301, DQB1*0101	DQB1*0501	DQB14
DQB1*0201, DQB1*0603	DQB1*0201	DQB6
DQB1*0604, DQB1*0301	DQB1*0604	DQB15
DQB1*0301, DQB1*0502	DQB1*0301	DQB6
DQB1*0603, DQB1*0101	DQB1*0603	DQB6
DQB1*0603, DQB1*0101	DQB1*0501	DQB14
DQB1*0201, DQB1*0101	DQB1*0501	DQB14
DQB1*0201, DQB1*0302	DQB1*0201, DQB1*0302	DQB6**
DQB1*0201, DQB1*0502	DQB1*0201	DQB6

(†) In this Table we only show representative examples of haplotypic combinations lacking those alleles the primers are fully matched with, for reasons of simplicity (see Table IV). Whenever these primers were used in heterozygotes carrying the alleles they specifically recognize, these alleles were selectively amplified. Note that in the examples shown in the Table the non-conserved primers selectively amplified the templates closer in sequence to the primer. The DRB and DQB1 alleles composing these haplotypes are shown under "haplotypes". The selected alleles and the primers used are indicated in the two other columns. More than one individual

was tested for some of the heterozygote combinations listed in this Table.

\* DRB3\*0101 and DRB3\*0201 templates both have one mismatch with primer DRB117. Selection of DRB5\*0101 could be related to the differential positioning of the mismatch with respect to the primer.

\*\* A weaker DRB4\*0101 template was also observed.

\*\*\* Despite the presence of a mismatch between these two DQB1 alleles, primer DQB6 was not able to select either of them.

These primers were able to selectively amplify certain DRB templates in all the heterozygous combinations tested. In heterozygotes carrying the alleles these primers are matched with, these alleles were selectively amplified; in heterozygotes not carrying the alleles specifically recognized by the primers, the DRB templates which had the fewest base pair mismatches with the primers were selectively amplified in the PCR. Specific examples of the latter are shown in Table V and Figure 4. As shown in Table V, Type 2

- 10 primers could differentially amplify DRB transcripts from the combinations of allelic cDNAs that differ from each other in as few as one nucleotide substitution, provided that high stringency annealing conditions are used for the PCR (annealing at 55°C). For example, in the heterozygote combination DRW13/DRW8-Dw8.1, the DRB1\*0801 allele (3 mismatches with the primer) was selected over DRB1\*1301 and DRB3\*0101 genes (each has 4 mismatches with the primer) by the DRB16 oligonucleotide primer. Although DRB3\*0101 or DRB3\*0201 and DRB5\*0101 genes all harbour one mismatch with primer DRB17, this oligonucleotide selected the DRB5\*0101 sequence in a DRW11/DRW15 heterozygote (Table V). It is possible that the differential positioning of the mismatches within the sequence recognized by the oligonucleotide also has an influence on the stability of the primer/cDNA complex and hence on the outcome of the PCR.

The ability of non-conserved primers to select certain alleles in heterozygote combinations was also tested for DQB1 genes (Table V). As with DRB-specific Type 2 primers, the use of high temperatures (55°C) in the annealing step of the PCR was required for achieving the selective amplification of single DQB1 alleles in heterozygotes with non-conserved primers. For instance, when annealing of primer DQB6 was allowed to proceed at 37°C in cDNAs from a DQB1\*0301/DQB1\*0501 and a DQB1\*0201/DQB1\*0603 heterozygote, 10 both alleles in both heterozygotes were equally amplified. At 55°C, the allele with the most homologous sequence to the 5' primer, was amplified over the other in the PCR.

Combinations of alleles both differing from the primer in two nucleotides but in different relative position were also 15 differentially amplified with a non-conserved primer. For instance, primer DQB6 selected the DQB1\*0604 sequence in a DQB1\*0604/DQB1\*0502 heterozygote (Table V). Five nucleotides separate the two mismatches between the DQB1\*0604 allele and the DQB6 primer, whereas only two nucleotides separate the 20 mismatches between the DQB1\*0502 and the primer.

These results clearly indicate that the oligonucleotide primers annealing to polymorphic regions at the 5' end of the target cDNAs can be tailored to achieve a reproducible selective amplification of a limited number of 25 DRB or DQB templates in complex heterozygous combinations. Although the use of Type 1 primers allows the unambiguous sequencing of all possible DQA, DQB, DPA and DPB heterozygotes, such an approach will not give absolute discriminatory information for all DRB heterozygotes. We 30 have shown that the simultaneous use of Type 1 and Type 2 primers for DRB will permit the clear elucidation of even the most complex of all DRB heterozygote combinations. When DRB-SBT is used for typing purposes, we perform three Type 2-reactions (using DRB23, 24 and 25) simultaneously with a Type 35 1-reaction (Table II). The simultaneous use of these

reactions using these primers has the highest discriminatory power for complete DRB typing in a single run and allows the identification of novel sequence heterogeneity. Only one Type 1 reaction is required for DQB1, one for DQA1, one for 5 DPA1 and one for DPB1 (Table II).

#### EXAMPLE III

##### Determining Unknown HLA Type of Subjects by Direct Sequencing of the Second Exon of Class II Genes

Routine HLA typing of large populations of 10 individuals for sequence polymorphisms can be performed by the use of the methodology reported here which can also identify previously unknown allelic variants. Figures 2A and 2B show a flow-chart for the routine protocol used to 15 determine sequence allelism of individuals of unknown HLA types.

##### 1. Employment of Primer Combinations for cDNA, PCR and Direct Sequencing Using RNA as Initial Template

For synthesizing cDNA molecules, the present 20 invention provides single strand DNA anti-sense oligonucleotide primers that anneal to conserved regions of the gene mRNAs to be reverse transcribed, amplified and sequenced. These oligonucleotide primers include an 25 oligonucleotide sequence that: (1) anneals to a conserved region (codons 105 through 111) shared by all the alleles at all the DRB loci, the latter being DRB1, DRB3, DRB4 and DRB5, respectively (e.g., primer DRE20). Four simultaneous cDNA reactions (one per tube) are performed for DRB typing, all 30 using primer DRE20 (reactions A, B, C and D in Table II and Figure 2A); (2) anneals to a conserved region (codons 105 through 111) shared by all the alleles at the DQB locus (e.g. primer DQB7) (reaction E in Table II and Figure 2A); (3) 35 anneals to a conserved region (codons 147 through 157) shared by all the alleles at the DQA locus (e.g. primer DQA9)

(reaction F in Table II and Figure 2A); (4) anneals to a conserved region (codons 105 through 111) shared by all the alleles at the DPB locus (e.g. primer DPB11) (reaction G in Table II and Figure 2A); (5) anneals to a conserved region (codons 104 through 110) shared by all the alleles at the DPA locus (e.g. primer DPA14) (reaction H in Table II and Figure 2A); (6) anneals to a conserved region (codons 222 through 228) shared by all the alleles at the DPA locus (e.g. primer DPA19) (reaction I in Table II and Figure 2A). The specific oligonucleotides added to each of these reactions once the cDNA synthesis is done in order to amplify and sequence the products are indicated below as well as in Table II and in Figure 2.

To amplify cDNA molecules corresponding to each expressed DRB loci of each chromosome (DRB1 and DRB3 or DRB4 or DRB5, depending on the haplotype -isotypic complexity-), a conserved oligonucleotide primer which anneals to codons -32 to -26 (e.g. oligonucleotide DRB11) is added to one of the four tubes where the cDNA synthesis reactions corresponding to DRB genes took place. The combination of the cDNA synthesis reaction primer and the newly added conserved primer is used to amplify all the alleles at all DRB loci expressed by a given individual. Each of the remaining three tubes containing DRB cDNA products receives one of three different non-conserved oligonucleotides (also called Type 2) annealing to codons 7-13 (e.g. primer DRB23), 5-11 (e.g. primer DRB24), 6-13 (e.g. primer DRB25), respectively. Each non-conserved primer is designed to favor the amplification of cDNAs corresponding to different groups of alleles at the DRB1 locus. Comparison of the sequencing ladders generated by these four reactions allows complete and accurate interpretation of the sequences corresponding to each of the four possible DRB genes expressed by a given individual (one or two for each of the parental chromosomes).

For the DQB1 locus, a conserved oligonucleotide primer which anneals to codons 1-7 of the DQB cDNAs (e.g. primer DQB13) can be used for amplifying each of the DQB1 genes expressed in any given individual (one for each parental chromosome). In the case of the DQA1 locus, a conserved single strand DNA oligonucleotide primer useful for amplifying each of the DQA1 genes expressed in any given subject anneals to codons -10 to -4 of the DQA1 cDNA (e.g. primer DQA10). For the DPB1 locus, a conserved oligonucleotide (e.g. primer DPB10) annealing to codons -19 to -13, is used to amplify each of the expressed DPB1 genes in any given subject. For DPA1 locus, a conserved oligonucleotide (e.g. primer DPA15) annealing to codons -23 to -17, is used to amplify each of the expressed DPA1 genes in a given subject. In a separate reaction, conserved primer DPA18, annealing to codons 59-65 of the DPA1 cDNAs is used in combination with the cDNA primer DPA19 to amplify each of the expressed DPA1 genes in any individual. This second DPA1 reaction is targeted at a second polymorphic region of this gene.

Primers useful in direct sequencing the polymerase chain reaction products corresponding to DRB loci include an anti-sense oligonucleotide primer (e.g. DRB12) annealing to codons 87-94 of all the alleles at DRB loci; this primer is used for sequencing the products generated by the first of the four DRB reactions. For direct sequencing the polymerase chain reaction products generated with the other three DRB reactions, an anti-sense oligonucleotide annealing to codons 97-103 of all the alleles at DRB1 locus can be used (e.g. primer DRB30). The use of a different sequencing oligonucleotide in these three DRB reactions allows reading of downstream polymorphic regions of DRB1 genes not seen in the first DRB reaction which uses the example sequencing primer DRB12. Primers useful in direct sequencing the polymerase chain reaction products corresponding to DQB1

locus include an anti-sense oligonucleotide primer (e.g. DQB5) annealing to codons 78-83 of all the alleles at this locus. Direct sequencing of polymerase chain reaction products corresponding to DQA1 locus include an anti-sense oligonucleotide primer (e.g. DQA29) annealing to codons 88-95 of all the alleles at this locus. Direct sequencing of polymerase chain reaction products corresponding to DPB1 locus include a sense oligonucleotide primer (e.g. DPB13) annealing to codons 12/-5 of all the alleles at this locus. For direct sequencing of polymerase chain reaction products for the DPA1 reaction which used primers DPA14 and DPA15, an anti-sense oligonucleotide annealing to codons 88-94 of all the alleles at this locus can be used (e.g. primer DPA16). For direct sequencing of polymerase chain reaction products for the DPA1 reaction which used primers DPA19 and DPA18, an anti-sense oligonucleotide annealing to codons 214-220 of all the alleles at this locus can be used (e.g. primer DPA20).

## 2. Employment of Primer Combinations for PCR and Direct Sequencing Using DNA Templates

To amplify DNA molecules corresponding to each DRB loci of each chromosome a conserved anti-sense oligonucleotide primer annealing to base pairs 18-38 of intron 3 (e.g. oligonucleotide DRB1406) is added to each of four PCR reaction tubes (reactions S, V, T and U in Table II and Figure 2B). Each of these four tubes will receive a different additional oligonucleotide annealing to codons -4 to +3 (e.g. primer DRB22), to codons 7-13 (e.g. primer DRB23), 5-11 (e.g. primer DRB24), 6-13 (e.g. primer DRB25), respectively. The first reaction is used to amplify all the alleles at all DRB loci carried by a given individual. Each of the remaining three reactions is designed to favor the amplification of DNA corresponding to different groups of alleles at the DRB1 locus. As with RNA templates, comparison of the sequencing ladders generated by these four reactions

allows complete and accurate interpretation of the sequences corresponding to each of the four possible DRB genes expressed by a given individual (one or two for each of the parental chromosomes).

For the DQB1 locus, two conserved oligonucleotide primers which anneal to codons 88-94 (e.g. primer DQB932) and 11-17 (e.g. primer DQB931) or 1-7 (e.g. primer DQB13) can be used for amplifying each of the DQB1 genes carried by any given individual (one for each parental chromosome) (reaction W in Table II and Figure 2B). For the DPB1 locus, two conserved oligonucleotides (a primer, e.g. DPB14, annealing to base pairs -42 to -62 of intron 2, and a primer e.g. DPB15, annealing base pairs 39-59 of intron 3) are used to amplify each of the DPB1 genes carried by any given subject (reaction X in Table II and Figure 2B). For DPA1 locus a conserved oligonucleotide such as DPA10 (annealing to base pairs -69 to -50 of intron 2) and DPA11 (annealing to base pairs 55-71 of intron 3) are used to amplify each of the DPA1 genes carried by a given subject (reaction Y in Table II and Figure 2B).

Primers useful in direct sequencing the polymerase chain reaction products generated from DNA templates corresponding to DRB loci include an anti-sense oligonucleotide primer (e.g. DRB12) annealing to codons 87-94 of all alleles at DRB loci; this primer is used for sequencing the products generated by the first of the four DRB reactions. For direct sequencing the polymerase chain reaction products generated with the other three DRB reactions, a sense oligonucleotide annealing to codons 39-46 of all the alleles at DRB1 locus can be used (e.g. primer DRB1400). The use of a different sequencing oligonucleotide in these three DRB reactions allows reading of downstream polymorphic regions of DRB1 genes not seen in the first DRB reaction which uses the example sequencing primer DRB12. Primers useful in direct sequencing the polymerase chain

reaction products corresponding to DQB1 locus include an anti-sense oligonucleotide primer (e.g. DQB5) annealing to codons 78-83 of all the alleles at this locus. Direct sequencing of polymerase chain reaction products corresponding to DPB1 locus include an anti-sense oligonucleotide primer (e.g. DPB16) annealing to base pairs 1-21 of intron 3 of all the alleles at this locus. For direct sequencing of polymerase chain reaction products for the DPA1 reaction an anti-sense oligonucleotide annealing to codons 76-82 of all the alleles at this locus can be used (e.g. primer DPA12).

#### Procedure for Determining Unknown HLA Type

A subject of unknown HLA type, diseased or not, is to be typed for Class II HLA polymorphism. From 10 to 50 mL of peripheral blood are drawn. The peripheral blood mononuclear cells are prepared by centrifugation over Ficoll-Hypaque gradients. The cells are then lysed in guanidium isothiocyanate and total cellular RNA prepared using conventional methods (either by centrifugation on cesium chloride gradients, which lasts about 16 hours, or by the guanidium isothiocyanate-phenol-chloroform extraction method, which can be performed in less than 4 hours. See Gough, *supra* (1988); Johns et al., *Anal. Biochem.*, 180:276 (1989). Otherwise genomic DNA from these cells or other sources (hair, blood stains, sperm, etc.) can be prepared with conventional methods such as provided by Higuchi, R. in *PCR Technology*, Erlich, M. (ed.), Stockton Press:31 (1989). DQB1, DQA1, DRB (DRB1, DRB3/4/5), DPA1 and DPB1 cDNA molecules are synthesized from total RNA using locus-specific primers. Approximately, one microgram of RNA is reverse transcribed with MolVRT (reverse transcriptase) and DRB (CODR20), DQB (CODQB7), DQA (CODQA9), DPB (DPB11) and DPA (DPA14, DPA19) (optional) -specific non-sense primers in a 20 uL final volume reaction (30-60 minute incubation). The

reaction for each Class II gene is performed in a different tube, but they can be performed in the same tube if preferred. For routine purposes, four simultaneous reactions are performed for DRB, one for DQB, one for DQA1, one for DPB1, and two for DPA1 gene products.

Once these reactions are completed, the enzymatic amplification of the respective cDNA molecules is then performed by directly adding to the 20 uL reverse transcription reaction, the reagents needed for the amplification step. Alternatively, if DNA is used, the primer combinations used for the PCR are those shown in Table II herein (the anti-sense primers as well as the sense primers will be different). This includes the PCR reagents and appropriate conserved and non-conserved oligonucleotide primers. This example uses four reactions for DRB (tubes 1, 2, 3 and 4), one for DQB (tube 5), one for DQA (tube 6), one for DPB (tube 7), and two for DPA (tubes 8 and 9, respectively). Reactions 2, 3 and 4 incorporate primers DRB23, DRB24 and DRB25, respectively. For rapid typing (in less than 24 hours), the latter are the preferred combinations. Alternative combinations of the primers that can be used are shown in Table II.

Once completed, the reactions are spun-dialyzed for about 15 minutes using Centricon (Amicon, Ultrafree (millipore)) or similar columns to remove unincorporated primers and dNTPs. The retentate or one half of the recovered retentate for each reaction is then directly sequenced using Taq polymerase and the primers described in Table II for each combination of primers used in the cDNA/PCR reactions using P-32 end-labeled (10 minutes) locus-specific sequencing primers (35 minutes).

The sequencing reactions products are loaded on an acrylamide gel, electrophoresed in 2-3 hours and exposed to X-ray films for 4-12 hours. The gels are read and results from

51

gels are compared to nucleotide sequences corresponding to all possible alleles.

Comparisons can be made visually using the naked eye or using a personal computer and a software package including the nucleotide sequences of all alleles of all haplotypes and routines which indicate how the comparison is to be performed as well as subroutines which will allow identification of new allelic sequences.

-52-

# SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Santamaria, Pedro  
Boyce-Jacino, Michael T.  
Barbosa, Jose J.  
Rich, Stephen S.  
Faras, Anthony J.

10

(ii) TITLE OF INVENTION: DNA Sequence-Based HLA  
Typing Method

15 (iii) NUMBER OF SEQUENCES: 49

(iv) CORRESPONDENCE ADDRESS:

20 (A) ADDRESSEE: Merchant & Gould  
(B) STREET: 3100 Norwest Center  
(C) CITY: Minneapolis  
(D) STATE: Minnesota  
(E) COUNTRY: USA  
(F) ZIP: 55402

25 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch,  
720 Kb.  
(B) COMPUTER: Northgate 386  
(C) OPERATING SYSTEM: DOS 4.0  
(D) SOFTWARE: WordPerfect 5.0

(vi) CURRENT APPLICATION DATA:

35 (A) APPLICATION NUMBER: 07/665,960  
(B) FILING DATE: 06-MAR-1991  
(C) CLASSIFICATION:

(viii) ATTORNEY INFORMATION:

40 (A) NAME: Kowalchuk, Alan W.  
(B) REGISTRATION NUMBER: 31,535  
(C) REFERENCE/DOCKET NUMBER: 600.243US  
600.243WO

45

(xi) TELECOMMUNICATION INFORMATION:

50 (A) TELEPHONE: (612) 332-5300  
(B) TELEFAX: (612) 332-9081

55

## (2) INFORMATION FOR SEQUENCE ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

10 (ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

20 (A) NAME/KEY: Oligonucleotide Primer DQB7

(B) LOCATION: Anneals to codons 105 to  
 111 of the DQB1 transcript of HLA  
 class II

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

30 G GTC GTT GAG GGC CTC TGT CC 21

## (2) INFORMATION FOR SEQUENCE ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

40 (ii) MOLECULE TYPE: Genomic DNA

45 (iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

50 (A) NAME/KEY: Oligonucleotide Primer  
 DRB20

(B) LOCATION: Anneals to codons 105 to  
 111 of the DRB1, DRB3, DRB4 and DRB5  
 transcripts of HLA class II

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTG CTG CAG GGG CTG GGT CTT 21

## (2) INFORMATION FOR SEQUENCE ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 22 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

20 (ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer DQA9

35 (B) LOCATION: Anneals to codons 148 to  
 155 of the DQA1 transcript of HLA  
 class II

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGT CAG GTT ACT CAT CTT GAA G 22

## (2) INFORMATION FOR SEQUENCE ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

55

-55-

- (ii) MOLECULE TYPE: Genomic DNA
- (iv) ANTI-SENSE: no
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE: Synthetically Derived
- (ix) FEATURE:
- (A) NAME/KEY: Oligonucleotide Primer  
DBR13
- (B) LOCATION: Anneals to codons 1 to 7 of  
the DBR1 transcript of HLA class II
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
- AGA GAC TCT CCC GAG GAT TTC 21  
Arg Asp Ser Pro Glu Asp Phe  
1 5
- (2) INFORMATION FOR SEQUENCE ID NO: 5:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Genomic DNA
- (iv) ANTI-SENSE: no
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE: Synthetically Derived
- (ix) FEATURE:
- (A) NAME/KEY: Oligonucleotide Primer  
DBR22
- (B) LOCATION: Anneals to codons -4 to +3  
of the DBR1, DRB3, DRB4 and DRB5  
transcripts of HLA class II

-56-

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
- CTG GCT TTG GCT GGG GAC ACC 21  
Leu Ala Leu Ala Gly Asp Thr  
-1 1
- (2) INFORMATION FOR SEQUENCE ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Genomic DNA
- (iv) ANTI-SENSE: no
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE: Synthetically Derived
- (ix) FEATURE:
- (A) NAME/KEY: Oligonucleotide Primer  
DRB11
- (B) LOCATION: Anneals to codons -33 to -  
26 of the DRB1, DRB3, DRB4 and DRB5  
transcripts of HLA class II
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
- TG TTC TCC AGC ATG GTG TGT C 21  
Phe Ser Ser Met Val Cys Leu  
-30
- (2) INFORMATION FOR SEQUENCE ID NO: 7:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Genomic DNA
- (iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment  
 (vi) ORIGINAL SOURCE: Synthetically Derived  
 5 FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQA10  
 10 (B) LOCATION: Anneals to codons -10 to -4  
 of the DQA1 transcript of HLA class  
 II  
 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:  
 CTG TCC TCC GTG ATG ACC CC 20  
 Leu Thr Thr Val Met Ser Pro  
 -10 -5  
 20

## (2) INFORMATION FOR SEQUENCE ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 (ii) MOLECULE TYPE: Genomic DNA  
 35 (iv) ANTI-SENSE: yes  
 (v) FRAGMENT TYPE: Internal Fragment  
 (vi) ORIGINAL SOURCE: Synthetically Derived  
 40 FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQB932  
 45 (B) LOCATION: Anneals to codons 88 to 94  
 of the DQB1 transcript of HLA class  
 II  
 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:  
 TCG CCT CTG CAG GGT CGC GCG 21  
 55

## (2) INFORMATION FOR SEQUENCE ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 5 MOLECULE TYPE: Genomic DNA  
 10 (ii) ANTI-SENSE: no  
 (iv) FRAGMENT TYPE: Internal Fragment  
 15 (v) ORIGINAL SOURCE: Synthetically Derived  
 (ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQB931  
 20 (B) LOCATION: Anneals to codons 11 to 17  
 of the DQB1 transcript of HLA class  
 II  
 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:  
 TTT AAG GGC ATG TGC TAC TTC 21  
 Phe Lys Gly Met Cys Tyr Phe  
 15

## (2) INFORMATION FOR SEQUENCE ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 40 MOLECULE TYPE: Genomic DNA  
 45 (ii) ANTI-SENSE: yes  
 (iv) FRAGMENT TYPE: Internal Fragment  
 50 (v) ORIGINAL SOURCE: Synthetically Derived  
 (vi)

-59-

- (ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQB30  
 5 (B) LOCATION: Anneals to codons 97 to 104  
 of the DQB1 transcript of HLA class  
 II  
 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:  
 A TGG GGA GAT GGT CAC TGT GG 21
- 15 (2) INFORMATION FOR SEQUENCE ID NO: 11:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 25 MOLECULE TYPE: Genomic DNA  
 (iv) ANTI-SENSE: yes  
 (v) FRAGMENT TYPE: Internal Fragment  
 30 ORIGINAL SOURCE: Synthetically Derived  
 35 FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB30  
 (B) LOCATION: Anneals to codons 97 to 103  
 of the DRB1, DRB3, DRB4 and DRB5  
 transcripts of HLA class II  
 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:  
 AGG ATA CAC AGT CAC CTT AGG 21  
 45
- 50 (2) INFORMATION FOR SEQUENCE ID NO: 12:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18 base pairs  
 (B) TYPE: Nucleic Acid  
 55

-60-

- (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 5 MOLECULE TYPE: Genomic DNA  
 (iv) ANTI-SENSE: yes  
 (v) FRAGMENT TYPE: Internal Fragment  
 10 ORIGINAL SOURCE: Synthetically Derived  
 (ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer DQB5  
 15
- (B) LOCATION: Anneals to codons 78 to 83  
 of the DQB1 transcript of HLA class  
 II  
 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:  
 GTA GTT GTG TCT GCA CAC 18
- 30 (2) INFORMATION FOR SEQUENCE ID NO: 13:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear  
 35 MOLECULE TYPE: Genomic DNA  
 (iv) ANTI-SENSE: yes  
 (v) FRAGMENT TYPE: Internal Fragment  
 40 ORIGINAL SOURCE: Synthetically Derived  
 45 FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB12  
 50 (B) LOCATION: Anneals to codons 87 to 94  
 of the DRB1, DRB3, DRB4 and DRB5  
 transcripts of HLA class II  
 55

-61-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:  
G CCG CTG CAC TGT GAA GCT C 20

(2) INFORMATION FOR SEQUENCE ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
(A) NAME/KEY: Oligonucleotide Primer  
DQA29

(B) LOCATION: Anneals to codons 82 to 89  
of the DQA1 transcript of HLA class  
II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:  
CAC GGT TCC GGT AGC AGC GGT AG 23

(2) INFORMATION FOR SEQUENCE ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

-62-

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
(A) NAME/KEY: Oligonucleotide Primer  
DQA30

(B) LOCATION: Anneals to codons 19 to 24  
of the DQA1 transcript of HLA class  
II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:  
TAC GGT CCC TCT GGC CAG 18  
Tyr Gly Pro Ser Gln Gln 20

(2) INFORMATION FOR SEQUENCE ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
(A) NAME/KEY: Oligonucleotide Primer  
DRB1400

(B) LOCATION: Anneals to codons 38 to 45  
of the DRB1, DRB3, DRB4 and DRB5  
transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:  
G CGC TTC GAC AGC GAC GTG G 20  
Val Arg Phe Asp Ser Asp Val Gly 40 45

## (2) INFORMATION FOR SEQUENCE ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB1401

(B) LOCATION: Anneals to codons 98 to 104 of the DRB1\*0701-2 transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:  
 GAG GTG ACT GTG TAT CCT GAC 21  
 Glu Val Thr Val Tyr Pro Asp 100

(2) INFORMATION FOR SEQUENCE ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQB1402

(B) LOCATION: Anneals to codons 142 to 148 of the DRB1, DRB3, DRB4 and DRB5 transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:  
 GAT CAG GCC TGT GGA CAC CAC 21

## (2) INFORMATION FOR SEQUENCE ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DQB1403

(B) LOCATION: Anneals to codons 127 to 133 of the DRB1, DRB3, DRB4 and DRB5 transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:  
 CCG GAA CCA CCT GAC TTC AAT 21

(2) INFORMATION FOR SEQUENCE ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

5 ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

10 (A) NAME/KEY: Oligonucleotide Primer  
DOB1406

15 (B) LOCATION: Anneals to bp18-38 to  
intron 33 of the DRB1, DRB3, DRB4 and  
DRB5 transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

20 GCCAAGAGTG GCCCTCCAG C 21

25 (2) INFORMATION FOR SEQUENCE ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

35 (ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

40 (vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

45 (A) NAME/KEY: Oligonucleotide Primer  
DRB825

50 (B) LOCATION: Anneals to codons 79 to 85  
of the DRB1, DRB3, DRB4 and DRB5  
transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

55 AAC CCC GTA GTT GTG TCT GCA 21

(2) INFORMATION FOR SEQUENCE ID NO: 22:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

10 (ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

15 (v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

20 (ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DRB824

25 (B) LOCATION: Anneals to codons 1 to 7 of  
the DRB1, DRB3, DRB4 and DRB5  
transcripts of HLA class II

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGG GAC ACC CGA CCA CGT TTC 21  
Gly Ala Thr Arg Pro Arg Phe  
1 5

35 (2) INFORMATION FOR SEQUENCE ID NO: 23:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

45 (ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

50 (v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

55 (ix) FEATURE:

-67-

(A) NAME/KEY: Oligonucleotide Primer  
DPB10

(B) LOCATION: Anneals to codons -19 to -13 of the DPB1 transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:  
CGG ACA GTG GCT CTG ACG GCG 21  
Arg Thr Val Ala Leu Tyr Ala  
-15

(2) INFORMATION FOR SEQUENCE ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPB11

(B) LOCATION: Anneals to codons 105 to 111 of the DPB1 transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:  
GTT GTG GTG CTG CAA GGG CCC 21

(2) INFORMATION FOR SEQUENCE ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

-68-

(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPB12

(B) LOCATION: Anneals to codons 97 to 103 of the DPB1 transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:  
CTT GGA GGG GGA AAC ATT CAC 21

(2) INFORMATION FOR SEQUENCE ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPB13

(B) LOCATION: Anneals to codons -5 to +2 of the DPB1 transcript of HLA class II

-69-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

TA CTG ATG GTG CTG CTC ACA T  
 Leu Leu Met Val Leu Leu Thr Ser  
 -12 -5

(2) INFORMATION FOR SEQUENCE ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
 DPB14

(B) LOCATION: Anneals to bp-42/-46 to  
 intron 2 of the DPB1 transcript of  
 HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AGAGGAGAA AGAGGATTAG A 21

(2) INFORMATION FOR SEQUENCE ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

-70-

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
 DPB15

(B) LOCATION: Anneals to bp39-59 to  
 intron 3 of the DPB1 transcript of  
 HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCCCTGGGCA CGGGCCCGCG G 21

(2) INFORMATION FOR SEQUENCE ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
 DPB16

(B) LOCATION: Anneals to bp1-21 to intron  
 3 of the DPB1 transcript of HLA class  
 II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CGGCCCAAAG CCCTCACTCA C 21

(2) INFORMATION FOR SEQUENCE ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

-71-

(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

5 (ii) MOLECULE TYPE: Genomic DNA  
(iv) ANTI-SENSE: no  
(v) FRAGMENT TYPE: Internal Fragment  
(vi) ORIGINAL SOURCE: Synthetically Derived  
(ix) FEATURE:

10 (A) NAME/KEY: Oligonucleotide Primer  
DPB17

15 (B) LOCATION: Anneals to bp -6/-26 to  
intron 2 of the DPB1 transcript of  
HLA class II

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:  
CGCTCAGTC CGCCCCCTCC C 21

25 (2) INFORMATION FOR SEQUENCE ID NO: 31:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

30 (ii) MOLECULE TYPE: Genomic DNA  
(iv) ANTI-SENSE: yes  
(v) FRAGMENT TYPE: Internal Fragment  
(vi) ORIGINAL SOURCE: Synthetically Derived  
(ix) FEATURE:

35 (A) NAME/KEY: Oligonucleotide Primer  
DPA14

40 (B) LOCATION: Anneals to codons 104 to  
110 of the DPA1 transcript of HLA  
class II

45 (2) INFORMATION FOR SEQUENCE ID NO: 33:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

50 (ii) MOLECULE TYPE: Genomic DNA  
(iv) ANTI-SENSE: yes  
(v) FRAGMENT TYPE: Internal Fragment

55

-72-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:  
GTC AAT GTG GCA GAT GAG GGT 21

5 (2) INFORMATION FOR SEQUENCE ID NO: 32:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

10 (ii) MOLECULE TYPE: Genomic DNA  
(iv) ANTI-SENSE: yes  
(v) FRAGMENT TYPE: Internal Fragment  
(vi) ORIGINAL SOURCE: Synthetically Derived  
(ix) FEATURE:

15 (A) NAME/KEY: Oligonucleotide Primer  
DPA15

20 (B) LOCATION: Anneals to codons -17 to -  
23 of the DPA1 transcript of HLA  
class II

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:  
CAT ATC AGA GCT GTG ATC TTG 21

30 (2) INFORMATION FOR SEQUENCE ID NO: 33:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

35 (ii) MOLECULE TYPE: Genomic DNA  
(iv) ANTI-SENSE: yes  
(v) FRAGMENT TYPE: Internal Fragment

40

45

50

55

-73-

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA16(B) LOCATION: Anneals to codons 88 to 94  
of the DPA1 transcript of HLA class  
II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CTT GGG AAA CAC GGT CAC CTC 21

(2) INFORMATION FOR SEQUENCE ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA17(B) LOCATION: Anneals to codons -3 to -9  
of the DPA1 transcript of HLA class  
II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CTG CTG AGT CTC CGA GGA GCT 21  
Leu Leu Ser Leu Arg Gly Ala  
-5

-74-

(2) INFORMATION FOR SEQUENCE ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA10(B) LOCATION: Anneals to bp-69/-50 of  
intron 2 of the DPA1 transcript of  
HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CTCTAGCTTT GACCACTTGC 20

(2) INFORMATION FOR SEQUENCE ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA11

-75-

(B) LOCATION: Anneals to bp55-71 to intron 3 of the DPA1 transcript of HLA class II

(xi) 5 SEQUENCE DESCRIPTION: SEQ ID NO: 36:  
AGTCTGAGGG TGGCAGAGAG G 21

10 (2) INFORMATION FOR SEQUENCE ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) 25 ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA12

(B) LOCATION: Anneals to codons 76 to 82 of the DPA1 transcript of HLA class II

(xi) 35 SEQUENCE DESCRIPTION: SEQ ID NO: 37:  
GGC CTG AGT GTG GTT GGA ACG 21

45 (2) INFORMATION FOR SEQUENCE ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

-76-

(v) FRAGMENT TYPE: Internal Fragment

(vi) 5 ORIGINAL SOURCE: Synthetically Derived  
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA18

(B) LOCATION: Anneals to codons 59 to 65 of the DPA1 transcript of HLA class II

(xi) 15 SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CTG GCT AAC ATT GCT ATA TTG 21  
Leu Ala Asn Ile Ala Ile Leu 65

20

25 (2) INFORMATION FOR SEQUENCE ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) 40 ORIGINAL SOURCE: Synthetically Derived  
(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA19

(B) LOCATION: Anneals to codons 222 to 228 of the DPA1 transcript of HLA class II

(xi) 55 SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GGT CCC CTG GGC CCG GGG GTC 21

-77-

(2) INFORMATION FOR SEQUENCE ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA20

(B) LOCATION: Anneals to codons 214 to 220 of the DPAL transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:  
 GCC AGA ACG CAG AGA CTT TAT 21

(2) INFORMATION FOR SEQUENCE ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

-78-

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DPA21

(B) LOCATION: Anneals to codons 68 to 74 of the DPAL transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:  
 AAC TTG AAT ACC TTG ATC CAG 21  
 Asn Leu Asn Thr Leu Ile Gln 70

(2) INFORMATION FOR SEQUENCE ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DRB23

(B) LOCATION: Anneals to codons 7 to 13 of the DRB1 transcript of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:  
 TTC TTG CAG CAG GAT AAG TA 20  
 Phe Leu Gln Gln Asp Lys Tyr 10

-79-

## (2) INFORMATION FOR SEQUENCE ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:  
 CCA CGT TTC TTG GAG TAC TCT 21  
 Pro Arg Phe Leu Gly Tyr Ser  
 5 10

(2) INFORMATION FOR SEQUENCE ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB25

-80-

(B) LOCATION: Anneals to codons 6 to 13  
 of the DRB1 transcript of HLA class  
 II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:  
 T TTC TTG GAG CAG GTT AAA CA 21  
 Arg Phe Leu Glu Val Lys His  
 10 15

(2) INFORMATION FOR SEQUENCE ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:  
 (A) NAME/KEY: Oligonucleotide Primer  
 DRB16

(B) LOCATION: Anneals to codons 29 to 35  
 of the DRB1, DRB3, DRB4 and DRB5  
 transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:  
 ACA TGC ATC TAT AAC CAA GAG 21  
 Arg Cys Ile Tyr Asn Glu  
 30 35

(2) INFORMATION FOR SEQUENCE ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: Nucleic Acid

-81-

(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DRB17

(B) LOCATION: Anneals to codons 29 to 35  
of the DRB1, DRB3, DRB4 and DRB5  
transcripts of HLA class II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

AGA TAC TTC CAT AAC CAG GAG 21  
Arg Tyr Phe His Asn Gln Glu 35

30 (2) INFORMATION FOR SEQUENCE ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer DQB6

(B) LOCATION: Anneals to codons -8 to -2  
of the DQB1 transcript of HLA class  
II

55

-82-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

CTG AGC ACC CCA GTG GCT GAG 21  
Leu Ser Thr Pro Val Ala Glu -5

10 (2) INFORMATION FOR SEQUENCE ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: Internal Fragment

(vi) ORIGINAL SOURCE: Synthetically Derived

(ix) FEATURE:

(A) NAME/KEY: Oligonucleotide Primer  
DQB14

(B) LOCATION: Anneals to codons -8 to -2  
of the DQB1 transcript of HLA class  
II

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

CTG AGC TCC TCA CTG GCT GAG 21  
Leu Ser Ser Ser Leu Ala Glu -5

45 (2) INFORMATION FOR SEQUENCE ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Genomic DNA

(iv) ANTI-SENSE: no

-83-

(v) FRAGMENT TYPE: Internal Fragment  
 (vi) ORIGINAL SOURCE: Synthetically Derived  
 5  
 (ix) FEATURE:  
 10 (A) NAME/KEY: Oligonucleotide Primer  
 DQB15  
 (B) LOCATION: Anneals to codons -8 to -2  
 of the DQB1 transcript of HLA class  
 II  
 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:  
 CTG AGC ACC TCG GTG GCT GAG 21  
 Leu Ser Thr Ser Val Ala Glu  
 -5  
 20

Applicants state that the paper copy of the  
 above "Sequence Listing" Section of the present  
 25 application, and the computer readable form of the same  
 submitted therewith, are the same.

84

## WHAT IS CLAIMED:

1. A method for determining a major histocompatibility complex genotype of a subject in a sample containing subject nucleic acid comprising:
  - (a) isolating nucleic acid from said sample;
  - (b) amplifying said nucleic acid by polymerase chain reaction to generate sufficient polymerase chain reaction product for each allele of said gene locus to be sequenced, all of said alleles for each gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer and at least one non-conserved oligonucleotide primer;
  - (c) sequencing directly each polymerase chain reaction product for each allele at each gene locus of each chromosome with Taq polymerase and a conserved primer specific for each locus that is sequenced; and
  - (d) analyzing each sequenced polymerase chain reaction product to determine the genotype of said subject.
2. The method of claim 1 wherein said isolated nucleic acid is genomic DNA.
3. The method of claim 1 wherein said isolated nucleic acid is RNA and further comprises the following step prior to amplifying said nucleic acid:
  - (a) synthesizing cDNA molecules for each allele of each gene locus to be sequenced, wherein said

85

synthesis employs a locus-specific oligonucleotide primer that anneals to a conserved region of each allele of each said gene locus.

4. The method of claim 1 wherein said major histocompatibility genotype to be determined is a HLA Class II genotype.

5. The method of claim 4 wherein said Class II gene locus to be sequenced is DQB1.

6. The method of claim 4 wherein said Class II gene locus to be sequenced is DQA1.

7. The method of claim 4 wherein said Class II gene loci to be sequenced are DRB 1/3/4/5.

8. The method of claim 4 wherein said Class II gene loci to be sequenced is DPA 1.

9. The method of claim 4 wherein said Class II gene loci to be sequenced is DPB 1.

10. The method of claim 1 wherein analyzing said sequenced polymerase chain reaction product involves comparing the nucleotide sequence of each allele of each gene locus sequenced to known sequences for each such gene locus followed by comparing the sequence of each allele of each gene locus amplified with a conserved/non-conserved oligonucleotide primer pair to the nucleotide sequence of each allele of such gene locus amplified with a conserved oligonucleotide primer pair.

86

11. The method of claim 1 wherein analyzing each polymerase chain reaction product to determine genotype is conducted with a computer having a program including nucleotide sequences of all alleles of all haplotypes for HLA Class II loci.

12. The method of claim 1 wherein said amplifying cDNA molecules with said conserved oligonucleotide primer includes annealing said conserved oligonucleotide primer to said cDNA at about 37°C.

13. The method of claim 1 wherein said amplifying cDNA molecules with said non-conserved primer includes annealing said non-conserved primer to said cDNA at about 55°C.

14. A method for determining the Class II histocompatibility genotype of a subject in a sample containing subject nucleic acid comprising:

(a) isolating nucleic acid from said sample;

(b) amplifying said nucleic acid by polymerase chain reaction to generate sufficient polymerase chain reaction product for each allele of said Class II gene locus to be sequenced, all of said alleles for each Class II gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer pair and at least one of said alleles for each Class II gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer and at least one non-conserved oligonucleotide primer;

(c) sequencing directly each polymerase chain reaction product for each allele at each Class

87

II gene locus of each chromosome with Taq polymerase and a conserved primer specific for each Class II locus that is sequenced; and

(d) determining the genotype of said subject by comparing the nucleotide sequence of each allele at each Class II locus sequenced to known sequences for each such Class II locus followed by comparing the sequence of each allele of each Class II locus amplified with a degenerated oligonucleotide primer to the nucleotide sequence of each allele of such Class II locus amplified with a conserved oligonucleotide primer.

15. The method of claim 14 wherein said isolated nucleic acid is RNA and further comprises the following step prior to amplifying said nucleic acid:

(a) synthesizing cDNA molecules for each allele of each Class II gene locus to be sequenced, wherein said synthesis employs a locus-specific oligonucleotide primer that anneals to a conserved region of each allele of each said Class II gene locus.

16. A method for determining the Class II HLA genotype of a subject in a sample containing subject nucleic acid comprising:

(a) isolating total cellular RNA from said sample;

(b) synthesizing cDNA molecules for each allele of at least one Class II gene locus to be sequenced, wherein said synthesis employs a locus-specific oligonucleotide primer that anneals to a conserved region of each allele of each said Class II gene locus;

88

(c) amplifying said cDNA molecules by polymerase chain reaction to generate a polymerase chain reaction product for each allele of said Class II gene locus to be sequenced, all of said alleles for each Class II gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer pair and at least one of said alleles for each Class II gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer and at least one non-conserved oligonucleotide primer; sequencing directly each polymerase chain reaction product for each allele at each Class II gene locus of each chromosome with Taq polymerase and a conserved primer specific for each Class II locus that is sequenced to produce a nucleic acid sequence ladder for each allele; and

(e) analyzing each nucleic acid ladder to determine the genotype of said subject by comparing the nucleotide sequence of each allele of each Class II locus sequenced to known sequences for each such Class II locus followed by comparing the sequence of each allele of each Class II locus amplified with a conserved/non-conserved oligonucleotide primer pair to the nucleotide sequence of each allele of such Class II locus amplified with a conserved oligonucleotide primer pair.

17. The method of claim 16 wherein said Class II HLA genotype to be determined includes nucleotide sequences for the DRB1, DRB3, DRB4, DRB5, DQB1, DQA1, DPA1 and DPB1 genes of said subject.

18. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 105 to 111 of the DQB transcript.

19. An oligonucleotide primer having the sequence  
GGTGGTTGAGGGCCCTCTGTCC. (SEQ. ID NO:1)

20. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 1 to 7 of DQB.

21. An oligonucleotide primer having the sequence  
AGAGACTCTCCGAGGATTC. (SEQ. ID NO:4)

22. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 148 to 155 of its DQA transcript.

23. An oligonucleotide primer having the sequence  
GGTGAGGTTACTGATCTTGAAG. (SEQ. ID NO:3)

24. An oligonucleotide primer comprising a single strand of DNA which anneals to codons -10 to -4 of DQA cDNAs.

25. An oligonucleotide primer having the sequence  
CTCTCCTCCGTGATGAGCCC. (SEQ. ID NO:7)

26. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 105 to 111 of DRB1 transcript.

27. An oligonucleotide primer having the sequence  
GTGCTGCAGGGGCTGGTCTT. (SEQ. ID NO:2)

28. An oligonucleotide primer comprising a single strand of DNA which anneals to codons -33 to -26 of DRB1 transcripts.

29. An oligonucleotide primer having the sequence  
TGTTCCTCCAGCATGGTGTGTC. (SEQ. ID NO:6)

30. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 7 to 13 of DRB1 transcript.

31. An oligonucleotide primer having the sequence  
TTCCTTGACGACGATAAGTA. (SEQ. ID NO:42)

32. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 5 to 11 of DRB 1 transcript.

33. An oligonucleotide primer having the sequence  
CCAGTTCTCTTGAGTACTCT. (SEQ. ID NO:43)

34. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 6 to 13 of DRB 1 transcript.

35. An oligonucleotide primer having the sequence  
TTTCTTGAGCAGGTTAACA. (SEQ. ID NO:44)

36. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 105 to 111 of DPB transcript.

37. An oligonucleotide primer having the sequence  
GTTGTGTGTCTGCAGGGCCC. (SEQ. ID NO:24)

91

38. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 222 to 228 of DPA transcript.

39. An oligonucleotide primer having the sequence  
GGTCCCTGGGCGGGGGTC. (SEQ. ID NO:39)

40. An oligonucleotide primer comprising a single strand of DNA which anneals to codons -19 to -13 of DPB transcript.

41. An oligonucleotide primer having the sequence  
CGGACAGTGGCTCTGACGGG. (SEQ. ID NO:23)

42. An oligonucleotide primer comprising a single strand of DNA which anneals to codons -23 to -17 of DPA transcript.

43. An oligonucleotide primer sequence of  
CATATCAGAGCTGTGATCTTG. (SEQ. ID NO:32)

44. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 59 to 65 of DPA transcript.

45. An oligonucleotide primer having the sequence  
CTGGCTAACATTGCTATATTG. (SEQ. ID NO:38)

46. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 104 to 110 of DPA transcript.

47. An oligonucleotide primer having the sequence  
GTCAATGTGGCAGATGAGGGT. (SEQ. ID NO:31)

92

48. An oligonucleotide primer having the sequence  
GCCGCTGCACCTGTGAAGCTC. (SEQ. ID NO:13)

49. An oligonucleotide primer having the sequence  
CCTGGAGGGGAAACATTAC. (SEQ. ID NO:11)

50. An oligonucleotide primer having the sequence  
GTAGTTGTGTCTGCACAC. (SEQ. ID NO:12)

51. An oligonucleotide primer having the sequence  
CACGTTCCGCTAGCAGCGGTAG. (SEQ. ID NO:14)

52. An oligonucleotide primer having the sequence  
CCTGGAGGGGAAACATTAC. (SEQ. ID NO:25)

53. An oligonucleotide primer having the sequence  
CTTGGAAACACGCTCACCTC. (SEQ. ID NO:33)

54. An oligonucleotide primer having the sequence  
GCCAGAACGCAGAGACTTTAT. (SEQ. ID NO:40)

55. An oligonucleotide primer comprising a single strand of DNA which anneals to base pairs 18 to 38 of intron 3 of DRB loci.

56. An oligonucleotide primer having the sequence  
of GCCAAGAGTGGCCTCGCAGC. (SEQ. ID NO:20)

57. An oligonucleotide primer comprising a single strand of DNA which anneals to codons -4 to +3 of the DRB transcript.

58. An oligonucleotide primer having the sequence  
CTGGCTTGGCTGGGGACACC. (SEQ. ID NO:5)

59. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 88 to 94 of the DQB transcript.

60. An oligonucleotide primer having the sequence  
TCGCTCTGTCAGGTCGCGG. (SEQ. ID NO:8)

61. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 11 to 17 of the DQB transcript.

62. An oligonucleotide primer having the sequence  
of TTTAAGGGCATGTGCTACTTC. (SEQ. ID NO:9)

63. An oligonucleotide primer comprising an single strand of DNA which anneals to base pairs -42 to -62 of intron 2 of the DPB locus.

64. An oligonucleotide primer having the sequence  
AGAGGGAGAAAGAGGATTAGA. (SEQ. ID NO:27)

65. An oligonucleotide primer comprising a single strand of DNA which anneals to intron 39 to 59 of intron 3 of the DPB gene.

66. An oligonucleotide primer having the sequence  
GCCCTGGCACGGGCCCGCGG. (SEQ. ID NO:28)

67. An oligonucleotide primer comprising a single strand of DNA which anneals to base pairs -69 to -50 of intron 2 of the DPA1 locus.

68. An oligonucleotide primer having the sequence  
CTCTAGCTTTGACCACCTTGC. (SEQ. ID NO:35)

69. An oligonucleotide primer comprising a single strand of DNA which anneals to base pairs 55 to 71 of intron 3 of the DPA1 locus.

70. An oligonucleotide primer having the sequence  
AGTCGAGGGTGCAGAGAGG. (SEQ. ID NO:36)

71. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 87 to 94 of the DRB transcript.

72. An oligonucleotide primer comprising a single strand of DNA which anneals to codons 38 to 45 of the DRB transcript.

73. An oligonucleotide primer having the sequence  
GCGCTTCGACAGCGACGTGG. (SEQ. ID NO:16)

74. An oligonucleotide primer having the sequence  
CGGCCCAAGCCCTCACTCAC. (SEQ. ID NO:29)

75. An oligonucleotide primer having the sequence  
GGCCTGAGTGTGGTTGGAACG. (SEQ. ID NO:37)

76. An oligonucleotide primer having the sequence  
TACTGAGGTGCTGCTCACAT. (SEQ. ID NO:26)

77. An oligonucleotide primer having the sequence  
CGCTCATGTCCGCCCTCC. (SEQ. ID NO:30)

78. An oligonucleotide primer having the sequence  
CTGCTGAGTCTCCGAGGAGCT. (SEQ. ID NO:34)

79. An oligonucleotide primer having the sequence  
AACTTGAATACCTTGATCCAG. (SEQ. ID NO:41)

95

80. An oligonucleotide primer having the sequence  
ATGGGGAGATGCTCACTGTGG. (SEQ. ID NO:10)

81. An oligonucleotide primer having the sequence  
TACGGTCCTCTGGCCAG. (SEQ. ID NO:15)

82. A method for rapid automated determination of  
major histocompatibility complex class genotype of a subject  
in a sample containing subject nucleic acid comprising:

- (a) isolating nucleic acid from said sample with an  
RNA/DNA extractor;
- (b) amplifying said nucleic acid by polymerase  
chain reaction using a thermocycler to generate  
a polymerase chain reaction product for each  
allele of each gene locus to be sequenced, all  
of said alleles for each gene locus and  
chromosome to be sequenced being amplified with  
at least one conserved oligonucleotide primer  
pair and at least one of said alleles for each  
gene locus and chromosome to be sequenced being  
amplified with at least one conserved  
oligonucleotide primer and one non-conserved  
oligonucleotide primer;
- (c) sequencing directly each polymerase chain  
reaction product for each allele at each gene  
locus of each chromosome in an automated  
sequencing apparatus with Taq polymerase and a  
conserved primer specific for each locus to be  
sequenced; and
- (d) analyzing each sequenced polymerase chain  
reaction product to determine the genotype of  
said subject with a computer having a data base  
with allelic sequence information to compare  
the sequence of each allele of each gene locus

96

sequenced to known sequences for each such gene  
locus followed by comparing the sequence of  
each allele of each gene locus amplified with a  
conserved/non-conserved oligonucleotide primer  
pair to the nucleotide sequence of each allele  
of such gene locus amplified with a conserved  
oligonucleotide primer pair.

83. A method for determining the genotype at one or  
more polymorphic gene locus of a subject in a sample  
containing subject nucleic acid comprising:

- (a) isolating nucleic acid from said sample;
- (b) amplifying said nucleic acid by polymerase  
chain reaction to generate sufficient  
polymerase chain reaction product for each  
allele of said gene locus to be sequenced, all  
of said alleles for each gene locus and  
chromosome to be sequenced being amplified with  
at least one conserved oligonucleotide primer  
pair and at least one of said alleles for each  
gene locus and chromosome to be sequenced being  
amplified with at least one conserved  
oligonucleotide primer and at least one non-  
conserved oligonucleotide primer;
- (c) sequencing directly each polymerase chain  
reaction product for each allele at each gene  
locus of each chromosome with a sequencing  
enzyme and a conserved primer specific for each  
locus that is sequenced; and
- (d) analyzing each sequenced polymerase chain  
reaction product to determine the genotype of  
said subject.

84. The method of claim 83 wherein said isolated  
nucleic acid is genomic DNA.

97

85. The method of claim 83 wherein said isolated nucleic acid is RNA and further comprises the following step prior to amplifying said nucleic acid:

- (a) synthesizing cDNA molecules for each allele of each gene locus to be sequenced, wherein said synthesis employs a locus-specific oligonucleotide primer that anneals to a conserved region of each allele of each said gene locus.

86. A method for rapid automated determination of the genotype at one or more polymorphic gene locus of a subject in a sample containing subject nucleic acid comprising:

- (a) isolating nucleic acid from said sample with an RNA/DNA extractor;
- (b) amplifying said nucleic acid by polymerase chain reaction using a thermocycler to generate a polymerase chain reaction product for each allele of each gene locus to be sequenced, all of said alleles for each gene locus and chromosome to be sequenced being amplified with at least one conserved oligonucleotide primer and one non-conserved oligonucleotide primer;
- (c) sequencing directly each polymerase chain reaction product for each allele at each gene locus of each chromosome in an automated sequencing apparatus with a sequencing enzyme and a conserved primer specific for each locus to be sequenced; and

98

- (d) analyzing each sequenced polymerase chain reaction product to determine the genotype of said subject with a computer having a data base with allelic sequence information to compare the sequence of each allele of each gene locus sequenced to known sequences for each such gene locus followed by comparing the sequence of each allele of each gene locus amplified with a conserved/non-conserved oligonucleotide primer pair to the nucleotide sequence of each allele of such gene locus amplified with a conserved oligonucleotide primer pair.

FIG. 1B

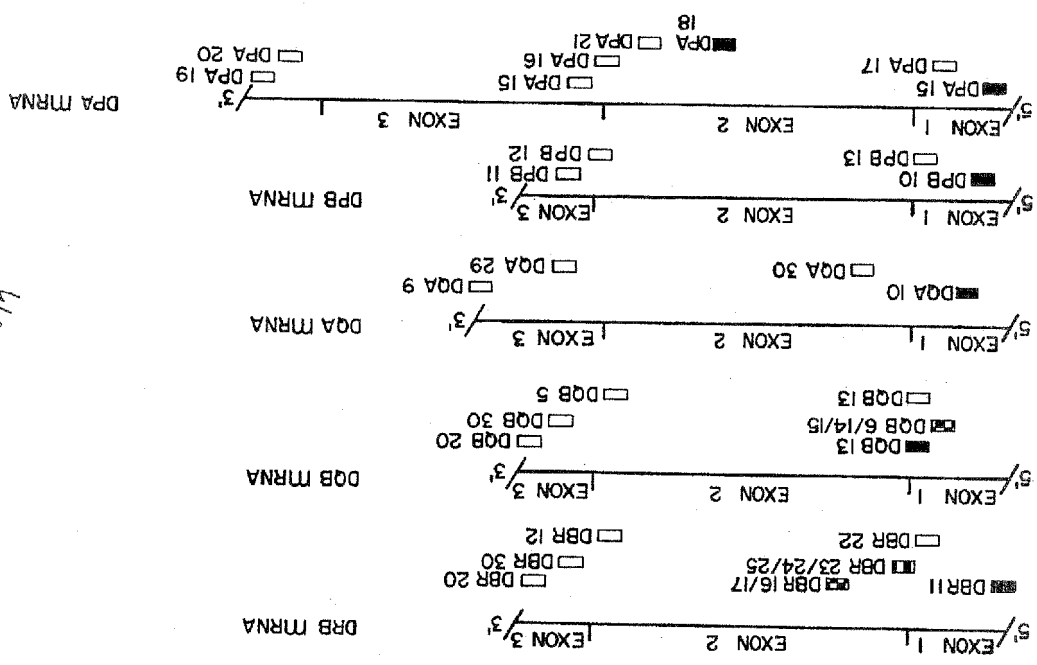


FIG. 1A

